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## Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome

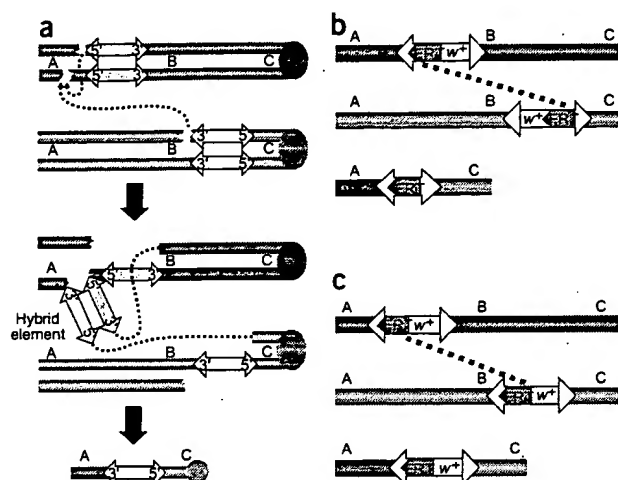
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In fruit fly research, chromosomal deletions are indispensable tools for mapping mutations, characterizing alleles and identifying interacting loci. Most widely used deletions were generated by irradiation or chemical mutagenesis. These methods are labor-intensive, generate random breakpoints and result in unwanted secondary mutations that can confound phenotypic analyses. Most of the existing deletions are large, have molecularly undefined endpoints and are maintained in genetically complex stocks. Furthermore, the existence of haplolethal or haplosterile loci makes the recovery of deletions of certain regions exceedingly difficult by traditional methods, resulting in gaps in coverage. Here we describe two methods that address these problems by providing for the systematic isolation of targeted deletions in the *D. melanogaster* genome. The first strategy used a *P* element-based technique to generate deletions that closely flank haploinsufficient genes and minimize undeleted regions. This deletion set has increased overall genomic coverage by 5–7%. The second strategy used FLP recombinase and the large array of FRT-bearing insertions described in the accompanying paper<sup>1</sup> to generate 519 isogenic deletions with molecularly defined endpoints. This second deletion collection provides 56% genome coverage so far. The latter methodology enables the generation of small custom deletions with predictable endpoints throughout the genome and should make their isolation a simple and routine task.

Deletion screens conducted at the Bloomington *Drosophila* Stock Center are based on the observation that *P* transposase often induces chromosomal aberrations involving the sites of two *P* insertions<sup>2</sup>. When two insertions are present at different sites on homologous chromosomes, deletions that remove the region between the two insertions can be recovered. In appropriately marked crosses, the deletions can be recognized from the recombination of visible, flanking markers. Our results are consistent with studies of *P* transposition showing that faulty transposition can produce deletions when the 5' end of one *P* element and the 3' end of a different *P* element are inserted in a chromosome as if they were the two ends of a single element<sup>3,4</sup> (Fig. 1a). This mistake in the normal transposition process has been called hybrid element insertion (HEI), because the paired 5' and 3' ends from different *P* elements inserted at a new chromosomal site by *P* transposase can be thought of as a temporary, synthetic or 'hybrid' *P* element<sup>3</sup>. Resulting deletions are flanked by one of the starting *P* constructs (Fig. 1a). This provides a convenient molecular tag for subsequent breakpoint characterization. Because a hybrid element can potentially insert anywhere in the genome, many different chromosomal rearrangements can result in addition to simple deletions. We isolated other rearrangements (data not shown) but specifically selected for deletions among recombinant progeny by complementation tests against mutations mapping between the original *P* insertion sites.

The success of the approach depends on a strong inherent bias of hybrid elements to insert in or near one of the original *P* insertions. If HEI were random, only one deletion breakpoint would be predictable.

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**Figure 1** Schematics for deficiency generation. The starting pairs of chromosomes are shown in orange and blue, with the transposon insertion elements indicated in light yellow or light blue and FRT elements in green. The order of theoretical markers A, B and C along the chromosomes is indicated. (a) The HEI strategy used at Bloomington. (b) Using FLP-FRT recombination to generate *w*<sup>-</sup> deficiencies from Exelixis transposon insertions. (c) Using FLP-FRT recombination to generate *w*<sup>+</sup> deficiencies from Exelixis transposon insertions.

We recovered only two deletions in all our screens in which the breakpoints differed from the sites of the original *P* insertions at the level of polytene cytology (*Df(2R)BSC25* and one not shown). The basis of this bias is unknown, but it resembles, at least superficially, the bias of single *P* elements to transpose locally<sup>5</sup>. Although the deletion endpoints are not entirely predictable, the method is useful because deletions can be targeted to particular genomic regions, the screens are moderately efficient and the size of deletions seems to be limited only by aneuploidy effects.

Figure 2a shows the crosses we used in HEI deletion screens and Table 1a gives markers and stocks we found particularly useful. When flanking markers and *P* insertions are linked (Fig. 2a), deletions will be recovered among progeny with both recessive marker phenotypes. With different linkage relationships, deletions will be recovered among progeny with different marker combinations. We recovered deletions with the two *P* insertions in the same and in opposite orientations. As *P* element ends are the only *cis*-acting sequences required for transposition, it seems that any two *P* constructs can be combined to produce

HEI deletions. We successfully used combinations of *P{PZ}*, *P{lacW}*, *P{SUPor-P}*, *P{EP}* and *P{GT1}* insertions.

So far, Bloomington has recovered 45 unique deletions using the HEI approach with *trans*-heterozygous *P* insertions (Supplementary Table 1 online). Table 2a summarizes their contribution to genomic coverage. All the screens were designed to fill gaps in deletion coverage, and most were designed to extend from a point adjacent to a haploinsufficient locus into an existing deletion. The fact that most haploinsufficient genes in *D. melanogaster* encode protein components of ribosomes<sup>6</sup> simplified the planning. By minimizing the undeleted regions to the small intervals between the two *P* insertions most closely linked to haploinsufficient, we could maximize coverage without the need for chromosomal duplications. For example, *Df(2L)BSC32* and *Df(2L)BSC36* flank the haploinsufficient locus *RpL9*, leaving only it and four other genes undeleted. So far, we have maximized deletion coverage around 14 known or potential haploinsufficient and maximized coverage to one side of an additional 10 haploinsufficient.

This method may be useful in certain experimental situations where *P* insertions are favorably located, but the method described below will probably prove more useful in most situations and will be used at Bloomington for further efforts to improve the Stock Center deletion collection. As described by Thibault *et al.*<sup>1</sup> in this issue of *Nature Genetics*, investigators at Exelixis generated a large isogenic collection of *P* and *piggyBac* insertion lines containing FRT sites. In the presence of FLP recombinase, FRT-bearing transposon insertions can be used to efficiently isolate deletions with precisely defined endpoints<sup>7</sup>. We used this method to generate a set of isogenic deletions as described below. The DrosDel Consortium is using a similar method (see URL).

**Table 1** Stock genotypes used in the generation of deletions

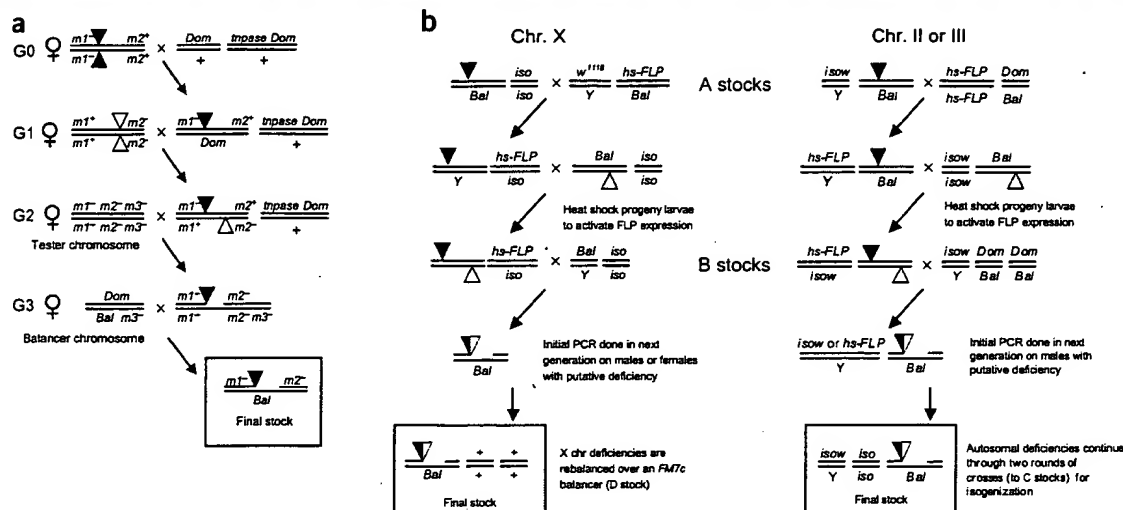
(a) Useful genotypes for generating *P*-element transposase-based deletions

Deletion in chromosome region	Useful flanking markers	Tester chromosome genotype	Transposase stock genotype	Balancer chromosome
21A-43E	<i>net<sup>1</sup></i> and <i>cn<sup>1</sup></i>	<i>net<sup>1</sup> b<sup>1</sup> cn<sup>1</sup> sp<sup>1</sup></i>	<i>T(2;3)ap<sup>2a</sup>, ap<sup>2a</sup>/CyO, H(PDelta2-3)HoP2.1, Cy<sup>1</sup>; Sb<sup>1</sup></i>	<i>SM1, SM5, SM6a</i> or <i>SM6b</i> : all marked with <i>Cy</i> , <i>cn</i> and <i>sp</i> mutations
25A-43E	<i>dp<sup>av1</sup></i> and <i>cn<sup>1</sup></i>	<i>dp<sup>av1</sup> cn<sup>1</sup> bw<sup>1</sup></i>	<i>T(2;3)ap<sup>2a</sup>, ap<sup>2a</sup>/CyO, H(PDelta2-3)HoP2.1, Cy<sup>1</sup>; Sb<sup>1</sup></i>	<i>SM6a, Cy<sup>1</sup> dp<sup>av1</sup> cn<sup>2P</sup> sp<sup>2</sup> bw<sup>1</sup></i>
43E-59E	<i>cn<sup>1</sup></i> and <i>bw<sup>1</sup> sp<sup>1</sup></i>	<i>cn<sup>1</sup> bw<sup>1</sup></i>	<i>T(2;3)ap<sup>2a</sup>, ap<sup>2a</sup>/CyO, H(PDelta2-3)HoP2.1, Cy<sup>1</sup>; Sb<sup>1</sup></i>	<i>SM6a, Cy<sup>1</sup> dp<sup>av1</sup> cn<sup>2P</sup> sp<sup>2</sup> bw<sup>1</sup></i>
62A-93D	<i>rho<sup>av1</sup></i> and <i>e<sup>1</sup></i>	<i>rho<sup>av1</sup> p<sup>o</sup> e<sup>1</sup></i>	<i>wg<sup>Sp-1</sup>/CyO, Cy<sup>1</sup>; TMS, P(Delta2-3)99B, Sb<sup>1</sup>/TM6, Ubx<sup>P15</sup></i>	<i>TM2, Ubx<sup>130</sup> p<sup>o</sup> e<sup>1</sup> or TM3, Sb<sup>1</sup> p<sup>o</sup> e<sup>1</sup></i>
73A-99B	<i>st<sup>1</sup></i> and <i>ca<sup>1</sup></i>	<i>st<sup>1</sup> Sb<sup>av1</sup> e<sup>1</sup> ro<sup>1</sup> ca<sup>1</sup></i>	<i>wg<sup>Sp-1</sup>/CyO, Cy<sup>1</sup>; TMS, P(Delta2-3)99B, Sb<sup>1</sup>/TM6, Ubx<sup>P15</sup></i>	<i>TM2, TM3, TM6, TM6B</i> or <i>TM6C</i> : all carry <i>ebony</i> and dominant markers

(b) Useful genotypes for generating FLP-FRT-based deletions

	Deletions on X	Deletions on 2nd	Deletions on 3rd
A stocks	<i>w<sup>1118</sup>, MKRS, M(3)76A, kar<sup>1</sup> ry<sup>2</sup> Sb<sup>1</sup>, P(hsFLP)86E/TM6B, Antp<sup>Hu</sup> e<sup>1</sup> Tb<sup>1</sup></i>	<i>P(hsFLP)1, w<sup>1118</sup>, Adv<sup>1</sup>/CyO, Cy<sup>1</sup> dp<sup>av1</sup> pr<sup>1</sup> cn<sup>2</sup></i>	<i>P(hsFLP)1, y<sup>1</sup> w<sup>1118</sup>, D<sup>TM6</sup>/TM3, kn<sup>1</sup> p<sup>o</sup> sep<sup>1</sup> I(3)89Aa<sup>1</sup> Ubx<sup>34a</sup> e<sup>1</sup> Sb<sup>1</sup></i>
B stocks	<i>XPG-L<sup>a</sup>/In(1)sc<sup>51L</sup>sc<sup>8R</sup>+dl-49, y<sup>4</sup> sc<sup>8</sup> sc<sup>51</sup> w<sup>1</sup> sr<sup>2</sup> B<sup>1</sup>; iso2; iso3</i>	<i>iso<sup>o</sup> w<sup>1118</sup>, wg<sup>Sp-1</sup>/CyO, Cy<sup>1</sup> dp<sup>av1</sup> pr<sup>1</sup> cn<sup>2</sup>; Ly<sup>1</sup>/TM6B, Antp<sup>Hu</sup> e<sup>1</sup> Tb<sup>1</sup></i>	<i>iso w<sup>1118</sup>, wg<sup>Sp-1</sup>/CyO Cy<sup>1</sup> dp<sup>av1</sup> pr<sup>1</sup> cn<sup>2</sup>; Ly<sup>1</sup>/TM6B, Antp<sup>Hu</sup> e<sup>1</sup> Tb<sup>1</sup></i>
C stocks		<i>iso w<sup>1118</sup>, wg<sup>Sp-1</sup>/CyO, Cy<sup>1</sup> dp<sup>av1</sup> pr<sup>1</sup> cn<sup>2</sup>; iso3</i>	<i>iso w<sup>1118</sup>, iso2; Dr<sup>1</sup>/TM6B, Antp<sup>Hu</sup> e<sup>1</sup> Tb<sup>1</sup></i>
D stock	<i>XPG-L<sup>a</sup>/FM7c, y<sup>31d</sup> sc<sup>8</sup> w<sup>o</sup>, sn<sup>12</sup> v<sup>o1</sup> g<sup>1</sup>; +; +</i>		

<sup>a</sup>XPG-L is an uncharacterized *P* lethal. <sup>b</sup>iso refers to an Exelixis isogenized chromosome.



**Figure 2** Genetic crossing schemes used to generate deletions. (a) Genetic scheme used for P-element transposase-based deletions. The crosses place P insertions (triangles) marked with flanking visible marker mutations ( $m1^-$  and  $m2^-$ ) in males together with a source of P transposase ( $tnpase$ ), so that hybrid element insertions can occur in premeiotic germ cells. Deletion events accompanied by recombination of flanking visible markers are identified in crosses to females carrying appropriate tester chromosomes. Deletions are established in stocks with balancer chromosomes ( $Bal$ ).  $Dom$  and  $m3^-$  represent other convenient dominant and recessive markers, respectively. (b) Genetic scheme used for FLP-FRT-based deletions. Crosses place two FRT-bearing transposon insertions (triangles) in *trans* in the presence of heat shock-driven FLP recombinase ( $hs-FLP$ ). Activation of FLP recombinase results in the generation of deletions that can be detected by PCR. Deletions are established in isogenized stocks with balancers ( $Bal$ ). Genotypes for nontransposon stocks (A, B, C, and D stocks) are provided in Table 1.  $Dom$ , dominant visible marker mutation;  $iso$ , isogenized chromosome; Y, Y chromosome.

The Exelixis deletion series is based on three of the four classes of transposon constructs described in the accompanying paper<sup>1</sup>. These transposon insertions contain FRT sites of 199 bp either 5' (in XP and WH transposons) or 3' (in RB transposons) of the *white+* transgene ( $w+$ ; Fig. 3a). In the presence of FLP recombinase, efficient

*trans*-recombination between FRT elements results in a genomic deletion with a single residual element tagging the deletion site (Fig. 1b,c). In deletions generated using XPs, which contain tandem FRT sites, the *cis*-recombination event occurs earlier than that in *trans*. Depending on the pair of starting insertions, their genomic orientation and the relative position of  $w+$  with respect to the FRTs, some deletions can initially be detected in the progeny by a loss of the  $w+$  marker (Table 3). The crosses outlined in Figure 2b and Table 1b allow the efficient recovery of deletions in four generations. Progeny are screened for the presence of the residual element by PCR detection of a resulting hybrid element (e.g.,  $XP:WH$ ) using element-specific primers, or by detection of residual element ends using paired element-specific and genome-specific primers (Fig. 3b). Final confirmation of the deletion ends can be provided by PCR or sequencing with genome-specific primers. In general, PCR screening of five  $w-$  individual progeny from  $w-$  deletion generation crosses, or 50 progeny from  $w+$  deletion generation crosses, resulted in four confirmed deletions. In addition to deletions, tandem duplications could also be isolated using these insertions and an appropriately designed PCR strategy.

Supplementary Table 2 online provides a list of 519 deletion stocks, covering approximately 56% of CG isoforms in the genome (see Table 2b), which have been transferred to the Bloomington *Drosophila* Stock Center. These deletions average 140 kb in length (ranging from 4.5 to 659 kb) and delete an average of 25 CG isoforms (ranging from 1 to 83). The density of starting insertions in the Exelixis collection and the precise nature of deletion endpoints allow the design of very small customized deletions. For example, one can generate null alleles for single genes for which a null point mutant is lacking (see examples in Supplementary Table 2 online: *Df(2L)Exel9033*, *Df(2R)Exel6062*, *Df(3L)Exel9028* and *Df(3R)Exel9056*). This targeted strategy can be also used to exclude problematic

**Table 2** Genome coverage provided by *D. melanogaster* deletion sets

(a) Genome coverage at Bloomington before acquiring Exelixis collection			
	Polytene band coverage of all Bloomington deletions (%)	Polytene band coverage added by Bloomington screens (%)	
X	86–93	0	
2L	93–97	5–11	
2R	93–95	8–13	
3L	91–93	3–6	
3R	90–93	6	
4	42–62	0	
Entire genome	90–94	5–7	
(b) Genome coverage provided by Exelixis isogenic deletion collection			
	Coverage of Flybase v3.1 transcription units (CGs)* (%)	Mean number of v3.1 transcription units (CGs) per deletion*	Mean deletion size (kb)
X	27	19	124.4
2L	80	24	154.5
2R	58	28	131.6
3L	47	22	133.8
3R	65	25	140.1
4	0	0	0
Entire genome	56	25	140

\*Transcription units include all splice forms given unique designations by Flybase (at the time of analysis, 18,109 CGs and CG splice forms were given unique identifiers).





original *P* insertion site<sup>4</sup>, which were of no interest to us. We distinguished deletions extending between different *P* insertion sites from other recombinant chromosomes by complementation tests. We characterized deletions in polyploid chromosome preparations. In successful screens, an average of one in every four recombinant premeiotic clones was associated with a deletion extending from one *P* insertion site to the other, and a unique *P*-to-*P* deletion clone was detected in 1 of every 3,000 progeny. There was wide variation in the efficiency of screens: approximately one-third of the *P* insertion pairs produced no recombinant *P*-to-*P* deletions, despite repeated attempts. We attribute this variability to inherent differences in the ability of *P* insertions to participate in hybrid element insertions. *P* insertions may be poorly mobile and rarely give rise to recombinant deletions, or they may be extremely mobile, excise efficiently in the intermediate G1 generation (where a single *P* insertion is combined with a transposase source) and leave few intact insertions to participate in HEI events in the G2 generation. Consequently, we generally designed screens of ~25,000 progeny and repeated the screens twice more before switching to a different pair of *P* insertions.

Most deletions generated by *P* transposase in the presence of *trans*-heterozygous *P* insertions will be tagged with one of the original *P* constructs (Fig. 1a). There is, however, a class of *P*-to-*P* deletions that will not be flanked by a *P* construct. When the two original *P* insertions are present in the same relative orientation, deletions may also form by a DNA repair process called hybrid excision and repair, the rejoining of the broken chromatid ends left behind when the 5' and 3' ends of two different *P* elements excise and participate in a HEI event<sup>3</sup>. No *P* construct will be present at the junction point. Hybrid excision and repair deletions have been recovered in *trans*-heterozygous *P* element screens, but they seem to be generated less often than HEI deletions (A. Paré and J. Ewer, personal communication).

Two variations on the HEI deletion method have been successful. First, one can eliminate the process of marking *P* insertions with flanking visible markers and scoring recombinants by testing for deletions among progeny that have lost the internal marker carried by one of the *P* constructs if one has an efficient assay for distinguishing deletions from the larger number of simple excisions occurring in the intermediate G1 generation. Such an approach was previously reported<sup>6</sup>. Second, one can recover deletions between the sites of *P* insertions if two insertions are first recombined in *cis* by meiotic crossover<sup>9</sup>.

**Genetic crosses for generation of FLP-FRT deletions.** Figure 2b provides a schematic of genetic crosses we used for FLP-FRT deletion generation; genotypes used can be found in Table 1b. The following describes autosomal deletion screens (X-chromosome deletion crosses are similar; Fig. 2b). We carried out all crosses at 25 °C unless otherwise stated. We selected transposon elements flanking the region to be deleted (Table 3). Males carrying one element were mated with females carrying a FLP recombinase transgene. Progeny males carrying both the element and FLP recombinase were then mated to females carrying the second element. After 2 d, we subjected parents and progeny (progeny contain both the FRT-bearing elements in *trans* and FLP recombinase) to a 1-h heat shock by placing the bottles into a 37 °C water bath. We removed parents after 72 h of total egg-laying time and subjected the bottles to daily 1-h heat shocks for 4 d more. We raised progeny to adulthood, collected virgin females and crossed them to males containing marked balancer chromosomes. Individual progeny males (five *w*- males for a *w*- deletion; 50 males for a *w*+ deletion) were then crossed pairwise to virgin females to generate additional progeny for PCR confirmation analysis and to balance the stocks in an isogenic background.

As we were unable to maintain healthy balanced *FM7c* stocks in our isogenic background, most X chromosome deletions were stocked in a nonisogenized *FM7c* background (D stock, Table 1b). Because the X chromosomes are derived from the same isogenized X and the backgrounds of the X deletion stocks are similar, phenotypic comparisons between X chromosome deletions should still be

valid. Alternatively, one could balance X chromosome deletions with the isogenized B stock for X deletions (a *Binsincy* balancer in an isogenized background).

**PCR confirmation of FLP-FRT-based deletions.** We carried out all PCR reactions using purified genomic DNA from five homogenized flies obtained from each isolate line (for genomic DNA preparation, see **Supplementary Methods** online). In general, it was sufficient to test 5 putative lines for each *w*- deletion and 50 putative lines for each *w*+ deletion. We used three different PCR strategies for deletion confirmation. Figure 3b provides a schematic and description of these strategies. Table 3 lists the PCR confirmation strategies used for initial detection of the various deletion types. **Supplementary Methods** online provide transposon primer sequences and expected fragment sizes for these combinations, as well as additional primer pair sequences for 'two-sided' PCR that may be used for secondary confirmation.

For products longer than 3 kb (see **Supplementary Methods** online), we carried out PCR using TaKaRa La *Taq* (Takara Bio). For products less than 3 kb, we used AmpliTaq Gold (Applied Biosystems). We used a standard touchdown program with both systems. Genomic primers were chosen using an in-house primer design tool and made by Bioscience.

**URL.** DrosDel Consortium is available at <http://www.drosdel.org.uk/>.

**Note:** Supplementary information is available on the Nature Genetics website.

#### ACKNOWLEDGMENTS

We thank J. Roote, D. Gubb, D. Coulson, T. Morley and M. Ashburner for early development of the *trans*-heterozygous *P* element-deletion technique and for introducing it to Bloomington; K. Matthews for advice and support; N. Klitgord for additional bioinformatics analysis of Exelixis deletions; J. McLaughlin for help with figures; the Exelixis genome biochemistry team for sequencing support; and the fly genetics team and bioinformatics staff for hands-on work and sequence analysis of the Exelixis *D. melanogaster* knockout collection. The work at Bloomington was supported by grants from the US National Institutes of Health National Center for Research Resources and the US National Science Foundation Division of Biological Infrastructure to K.R.C. and T.C.K.

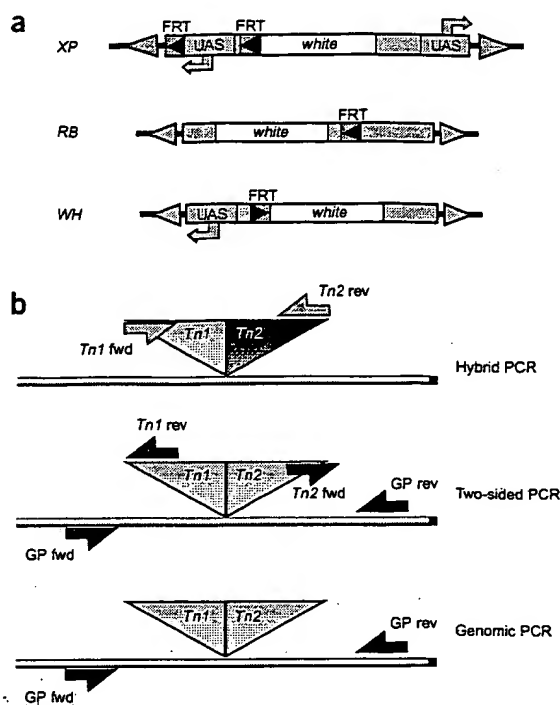
#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Genetics website for details).

Received 5 January; accepted 28 January 2004

Published online at <http://www.nature.com/naturegenetics/>

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**Figure 3** FRT-bearing transposons and PCR strategies used in FLP-FRT-based deletions. (a) Schematic of the three transposon types used in FLP-FRT-based deletions. FRT sites, UAS-containing sequences and the gene *white* are indicated. See Thibault *et al.*<sup>3</sup> for details. FRT orientation is indicated by the direction of the arrowheads (FRT sites must be in the same orientation for deletion generation). (b) For transposon combinations that generate a recombinant hybrid element (composed of two different element types), transposon-specific primers were used to generate a fragment of known size across the newly formed hybrid (hybrid PCR). For transposon combinations that regenerate an intact element, a genomic primer in combination with a transposon-specific primer for each end of the transposon was used (two-sided PCR). We also carried out PCR using only genomic primers for additional confirmation (genomic PCR). **Table 3** lists the PCR confirmation method used for initial detection of the various deletion types. **Supplementary Methods** online provides transposon primer sequences and expected fragment sizes.

complex differences in the genetic backgrounds of stocks, large individual deletion sizes and imprecisely defined endpoints.

In summary, we describe the highest resolution deletion coverage of a metazoan genome so far. Improvements to genome coverage and breakpoint distribution will be continued by the Bloomington *Drosophila* Stock Center using the FRT-based deletion strategy described in this manuscript, as well as by the DrosDel Consortium. Furthermore, the establishment of these methods and the widespread distribution of FRT-bearing insertion stocks<sup>1</sup> and tools allows individual scientists to generate customized aberrations in the *D. melanogaster* genome in an efficient and straightforward manner.

## METHODS

**P transposase-based deletion screens.** Visible marker mutations must first be combined in *cis* with each *P* insertion by standard meiotic crossover crosses. Once marked, the *P* insertion chromosomes may be used in the genetic scheme shown in Figure 2a. Many different visible markers could potentially be used, but Table 1a lists markers and stocks we found useful for various chromosomal intervals.

Recombinant chromosomes can arise either from HEI events involving two different *P* insertions or from events involving the two copies of a single *P* insertion present on sister chromatids. The latter yield small deletions flanking the

regions, such as haploinsufficient loci, or to generate precisely overlapping regions for high resolution coverage. Additionally, as deletions generated by this method are carried in the same isogenic background, meaningful comparisons between deletion-based modification phenotypes are relatively straightforward. We used this approach to refine regions of phenotypic interest by sequential deletion mapping (K.A.E., H.L.F.-L., M.B. Mahoney & A.L.P., unpublished data) and to carry out genome-wide screens for specific phenotypes (S. Thibault, personal communication). Previously available deletions made such analyses more difficult, owing to the

**Table 3** Allowable pairwise combinations of Exelixis transposons for deletion generation

Lower coordinate tn1 (strand)	Higher coordinate tn2 (strand)	w+/-	Initial PCR confirmation method	UAS remaining?
RB (-)	RB (-)	w+	Two-sided	No
RB (-)	WH (+)	w+	Hybrid	No
RB (+)	RB (+)	w+	Two-sided	No
RB (+)	WH (-)	w+	Hybrid	Yes
RB (+)	XP (+)	w+	Two-sided*	Yes
WH (-)	RB (+)	w+	Hybrid	No
WH (-)	WH (-)	w+	Two-sided	Yes
WH (-)	XP (+)	w+	Two-sided*	Yes
WH (+)	RB (-)	w+	Hybrid	Yes
WH (+)	WH (+)	w+	Two-sided	Yes
XP (-)	RB (-)	w+	Two-sided*	Yes
XP (-)	WH (+)	w+	Two-sided*	Yes
XP (-)	XP (-)	w+	Two-sided	Yes
XP (+)	XP (+)	w+	Two-sided	Yes
XP (+)	RB (+)	w-	Hybrid	No
XP (+)	WH (-)	w-	Hybrid	Yes
RB (-)	XP (-)	w-	Hybrid	No
WH (+)	XP (-)	w-	Hybrid	Yes

\*Hybrid PCR, though not tested by us, should also work for these deletion types.

# Mammalian MAP kinase signalling cascades

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**Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes, unique to eukaryotes, that are involved in many facets of cellular regulation. Initial research concentrated on defining the components and organization of MAPK signalling cascades, but recent studies have begun to shed light on the physiological functions of these cascades in the control of gene expression, cell proliferation and programmed cell death.**

**M**APKs are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. MAPKs also respond to chemical and physical stresses, thereby controlling cell survival and adaptation. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK)<sup>1</sup>. These modules may be activated by STE20 kinases or small GTP-binding proteins<sup>2</sup>. Many MAPKs activate specific effector kinases—MAPK-activated protein kinases (MAPKAPKs)—and are inactivated by MAPK phosphatases.

As the regulation of MAPK signalling has been discussed elsewhere<sup>1,2</sup>, we focus on recent progress in understanding MAPK function in mammals. Because of the ease of genetic analysis, the functions and regulation of MAPK cascades are best understood in budding yeast<sup>3</sup>, but the analysis of targeted mutations in mice and development of specific inhibitors have begun to shed light on their functions in mammals. It is becoming clear that MAPKs regulate almost all cellular processes, from gene expression to cell death.

## Mammalian MAPKs

Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 $\alpha$ / $\beta$ / $\gamma$ / $\delta$ ) and ERK5, that are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling. Presumably each MAPKKK confers responsiveness to distinct stimuli. For example, activation of ERK1/2 by growth factors depends on the MAPKKK c-Raf, but other MAPKKKs may activate ERK1/2 in response to pro-inflammatory stimuli.

The situation is quite complex with the JNK and p38 cascades, which respond to many stimuli and can be activated on overexpression of at least a dozen MAPKKKs, whose physiological functions and specificities remain elusive. Dominant-negative catalytically inactive MAPKKKs can be used to address these issues, but the results of such experiments can be confusing. For example, at least four MAPKKKs were implicated in JNK activation by tumour necrosis factor (TNF) and interleukin (IL)-1, including ASK1 (MEKK5), TAK1, MLK and MEKK1 (reviewed in ref. 4). These difficulties are probably generated by loss of specificity caused by overexpression of dominant-negative mutants.

Systematic genetic ablation of MAPKKKs may provide definitive answers to many of these questions. For instance, it has been shown that MEKK1 is required for JNK activation in embryonic stem cells or fibroblasts by microtubule-disrupting agents, lysophosphatidic acid, viral infection, double-stranded RNA and serum<sup>4,5</sup>. MEKK1 also is required for activation of JNK by TNF and IL-1 in embryonic stem cells<sup>4</sup>, but not in fibroblasts<sup>5</sup>. These experiments revealed that MEKK1 is dispensable for activation of JNK by ultraviolet radiation and protein-synthesis inhibitors and is not essential for ERK1/2 or

p38 activation. Such results suggest considerable specificity in MAPK activation even at the MAPKKK level.

## Specificity in MAPK activation and function

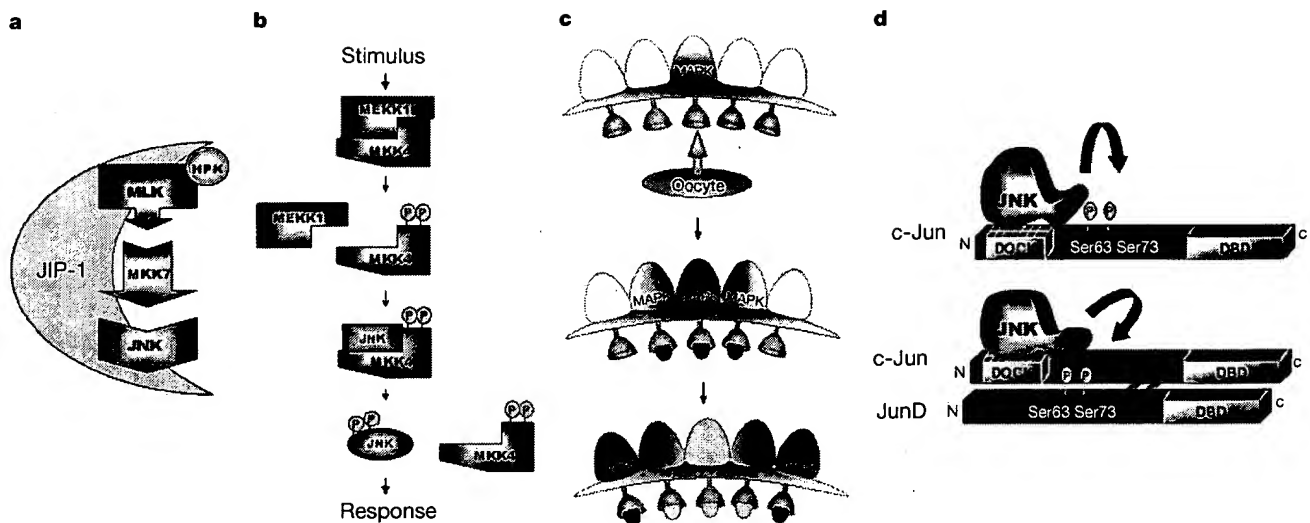
The control of diverse cellular processes in response to a plethora of extracellular stimuli by a few MAPKs implies that considerable specificity is built into MAPK activation and function. Several distinct but not mutually exclusive mechanisms secure specificity in MAPK activation. In yeast, the STE5 protein functions as a scaffold that organizes the three components of a pheromone-responsive MAPK cascade and its upstream activators into a specific module<sup>3</sup>. A search for analogous mammalian scaffolds led to JIP1, which organizes JNK1/2, MKK7 and the MAPKKK MLK1 into a specific signalling cassette (Fig. 1a)<sup>6</sup>. MP1, another mammalian scaffold protein not related to JIP-1, interacts with ERK1 and MEK1, thereby potentiating ERK1 activation<sup>7</sup>.

A second mechanism ensuring specific MAPK activation depends on sequential physical interactions between members of a given cascade<sup>8</sup>. For instance, JNK1/2 is bound by the N-terminal extension of MKK4 (JNKK1), which also interacts with the catalytic domain of MEKK1. Each interaction is disrupted on activation of the downstream kinase (Fig. 1b). Whereas scaffolds increase specificity at the expense of signal amplification, sequential interactions provide amplification without sacrificing specificity<sup>8</sup>. Both mechanisms may operate in parallel, leading to differential activation of the same MAPK in response to distinct stimuli.

A third and more complex mechanism for signal amplification is based on the ability of MAPKs to regulate indirectly the expression of both ligands and inhibitors for cell-surface receptors that feed into MAPK cascades<sup>9</sup>. Such positive and negative autocrine loops can generate specific spatial patterns of MAPK activation (Fig. 1c).

Once activated, MAPKs need to find their targets. Although it is necessary to limit the phosphorylation of irrelevant substrates, it is also important that each MAPK recognizes a number of substrates to allow the regulation of many processes. This problem seems to have been solved through a bipartite enzyme–substrate interaction. All MAPKs recognize similar phosphoacceptor sites composed of serine or threonine followed by a proline, and the amino acids that surround these sites further increase the specificity of recognition by the catalytic pocket of the enzyme. Full specificity is ensured through a docking interaction mediated by another site on the kinase that recognizes a distinct site on the substrate (Fig. 1d). As first described for c-Jun, a short sequence that precedes the two principal JNK phosphoacceptor sites, Ser 63 and Ser 73, is recognized by an interaction surface on JNK, outside its catalytic pocket<sup>10</sup>. This interaction determines the efficiency of c-Jun phosphorylation and the choice of phosphoacceptor sites (Fig. 1d). Similar interactions involving distinct phosphoacceptor and docking sites determine substrate recognition by other MAPKs<sup>11</sup>.

Spatial localization of signalling molecules further augments specificity in signal transduction. For instance, MEKK1 colocalizes with elements of the cytoskeleton<sup>1</sup>, and cytoskeletal rearrangements



**Figure 1** Mechanisms for generation of specificity in MAPK activation and function. **a**, JIP-1 acts as a scaffold that ties together the upstream kinase HPK-1, the MAPKKK MLK1, the MAPKK MKK7 and the MAPK JNK into a specific signalling module. **b**, Sequential and specific interaction between members of a MAPK cascade. MEKK1 interacts with inactive MKK4 to form a MEKK1–MKK4 complex. MEKK1 phosphorylates and activates MKK4, resulting in dissociation of the complex. The free and active MKK4 then specifically interacts with JNK. Once JNK is activated, the MKK4–JNK complex is disrupted and the active JNK is now freed to phosphorylate downstream effectors. **c**, Specific spatial patterns of MAPK activation by positive and negative autocrine loops in the *Drosophila* oocyte. A ligand called Gurken (red arrow) that is localized in the underlying oocyte activates a MAPK cascade (red gradient) in follicle cells, through an EGF receptor (green). This results in upregulation of another EGF receptor agonist, 'Spitz' (red), that activates MAPK in neighbouring cells. This is followed by expression of the inhibitory ligand 'Argos' (yellow) in midline cells with higher levels of MAPK activity, which eventually inhibits of MAPK activity in this region. **d**, The JNK docking region (DOCK) of c-Jun determines its specific phosphorylation at Ser 63 and Ser 73 by JNK. A substrate lacking a functional docking site, such as JunD, can be phosphorylated by JNK that is recruited through the DNA-binding domain (DBD) of c-Jun.

may stimulate MEKK1 activity<sup>4,5</sup>. Such localized JNK activation may allow MEKK1 to modulate cell motility<sup>4,5</sup>.

### General functions of MAPK cascades

One of the most explored functions of MAPK signalling modules is regulation of gene expression in response to extracellular stimuli<sup>12</sup>. JNKs phosphorylate Jun proteins and thereby enhance their ability to activate transcription without affecting DNA binding<sup>10</sup>. Most MAPKs phosphorylate Ets transcription factors that are involved in induction of *fos* genes, whose products heterodimerize with Jun proteins to form activation protein 1 (AP-1) complexes<sup>12</sup>. The p38 proteins phosphorylate and enhance the activity of MEF2C and related family members<sup>13</sup>. In all of these cases, the MAPKs function inside the nucleus and target transcription factors that are pre-bound to DNA. Most transcription factors that are regulated by MAPKs are dimers, and structural analysis indicates that some MAPKs dimerize on activation<sup>1</sup>; however, MAPK dimerization may facilitate phosphorylation of dimeric transcription factors.

Although transcription factors are important MAPK targets, only part of the active MAPK pool translocates to the nucleus and much remains in the cytoplasm and other subcellular compartments. Correspondingly, MAPKs also regulate gene expression through post-transcriptional mechanisms involving cytoplasmic targets. JNK, for instance, is involved in IL-2 messenger RNA stabilization in activated T cells<sup>14</sup>. The target for JNK action appears to be a cytoplasmic protein that is recruited to IL-2 mRNA through two other proteins that bind to the 5' untranslated region<sup>14</sup>. Other mRNA species are stabilized in response to p38 activation<sup>15,16</sup>. MAPKs are also involved in translational control, but in this case (as well as in some cases of mRNA stabilization<sup>15</sup>) their action is mediated by the MAPKAPK-2 and MNK1 effector kinases<sup>17,18</sup>. MNK1 phosphorylates translation initiation factor 4e (eIF-4e), augmenting the interaction of the cap-binding complex with capped mRNAs, thereby enhancing polysome assembly.

MAPKs regulate cell proliferation. ERK1/2 stimulates DNA synthesis through phosphorylation of carbamoyl phosphate synthetase

II, a rate-limiting enzyme in pyrimidine nucleotide biosynthesis<sup>19</sup>. In addition, the ERKs, probably through MAPKAPKs such as RSK, promote cell-cycle progression by inactivating MYT1, a cell-cycle inhibitory kinase<sup>20</sup>, but arrest meiotic cells at metaphase II by activating a cytostatic factor<sup>21,22</sup>. The ERKs can also stimulate cell proliferation indirectly by enhancing AP-1 activity, resulting in cyclin D1 induction<sup>23</sup>. That pathway alone is insufficient for initiation of DNA synthesis and needs assistance from phosphatidylinositol-3-OH kinase, which is indirectly activated by autocrine growth factors whose expression is ERK responsive<sup>23</sup>. Autocrine factors are important in cellular responses to MAPKs<sup>9</sup> and can provide a means for one MAPK cascade to activate other signalling pathways<sup>24</sup>. In T cells, the JNK pathway may stimulate proliferation by inducing IL-2 expression<sup>25</sup>.

MAPKs also control cell survival. By and large, activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are linked to induction of apoptosis<sup>26</sup>. This dichotomy, however, is an oversimplification, and the actual roles of each MAPK cascade are highly cell type and context dependent. A pro-apoptotic function has been established for prolonged JNK activation in certain neuronal cells, especially in the CA1 layer of the hippocampus after intense activation of glutamate receptors<sup>26–28</sup>. This process does not occur in *Jnk3*<sup>−/−</sup> mice<sup>28</sup> or in mice expressing a mutant form of c-Jun (c-Jun<sup>A63/73</sup>) lacking the JNK phosphoacceptor sites<sup>29</sup>; however, transient and more modest JNK activation in response to physiological stimulation does not result in neuronal apoptosis<sup>30</sup>. JNK-induced neuronal apoptosis partially depends on induction of death cytokines, such as Fas ligand, whose promoter contains an AP-1 site, explaining the dependence on c-Jun phosphorylation<sup>27,29</sup>. JNK1 and JNK2 are required for apoptosis of ultraviolet-irradiated fibroblasts, in which they have been suggested to activate directly the apoptotic machinery<sup>31</sup>. However, the combined JNK1/2 deficiency only prevents ultraviolet-induced apoptosis and does not hinder other pro-apoptotic stimuli<sup>31</sup>. c-Jun-deficient fibroblasts also exhibit specific resistance to ultraviolet-induced apoptosis<sup>32</sup>. Given that JNKs are vigorously activated after ultraviolet exposure and needed

for c-Jun induction<sup>31</sup>, it is likely that the pro-apoptotic function of JNK1/2 in ultraviolet-irradiated fibroblasts is mediated through c-Jun<sup>32</sup>. Nevertheless, in cells exposed to physiologically relevant doses of ultraviolet radiation, JNK-induced c-Jun stimulates cell-cycle re-entry rather than inducing apoptosis<sup>32</sup>.

The mechanism by which ERK protects cells from apoptosis is complex and Ras, a potent ERK activator, may also promote apoptosis<sup>33</sup>. In cerebellar granular cells, ERK activation by survival factors prevents apoptosis through RSK, which inactivates the pro-apoptotic protein BAD<sup>34</sup>. ERK may also induce growth factors that promote cell survival.

### *In vivo* analysis of MAPK and MAPKK function

Although much can be learned about MAPK functions from *ex vivo* studies, it is important to investigate both their normal and their pathophysiological functions in the whole animal. Such analysis, accomplished through generation of mutant mouse strains by gene targeting, is also useful for determining the organization and regulation of MAPK signalling cascades. As the different MAPKKs exhibit high levels of specificity, it should also be possible to learn about MAPK function by targeting relevant MAPKKs. Some of the findings and the conclusions derived from analyses of MAPK- and MAPKK-deficient mice are summarized in Table 1.

ERK1/2. Only the knockout of ERK1 has been described<sup>35</sup>. *Erk1*<sup>-/-</sup> mice are viable and appear normal and with a modest defect in T-cell development (positive selection)<sup>35</sup>. It is likely that most ERK1 functions are equally served by ERK2. A similar and more marked defect is present in transgenic mice expressing dominant-negative MEK1 in thymocytes<sup>36</sup>, and *Mek1*<sup>-/-</sup> mice die *in utero*, exhibiting defective placental vascularization<sup>37</sup>.

Despite impairment of positive selection and loss of ERK activity, thymocytes expressing dominant-negative MEK1 exhibit normal IL-2 induction and a mitogenic response<sup>36</sup>. Thus, other MAPKs are probably involved in IL-2 induction. As suggested by the *Mek1*<sup>-/-</sup> phenotype, MEK1 is required for migration of vascular endothelial cells as well as fibroblasts plated on fibronectin<sup>37</sup>. However, fibronectin-induced ERK activity remains normal<sup>37</sup>. An ERK1/2-MEK1 module may target specific substrates involved in cell migration while making only a small contribution to total ERK1/2 activity. JNKs. All three *Jnk* loci have been knocked out. On mixed genetic backgrounds, none of the mutations results in lethality or obvious defects. However, *Jnk1*<sup>-/-</sup>*Jnk2*<sup>-/-</sup> double mutants die at mid-gestation, exhibiting defective neural-tube closure<sup>38,39</sup>. Thus, JNK functions needed for development and viability are not isoform specific. Unexpectedly, deletion of MKK4 results in a more severe phenotype than the combined loss of JNK1/JNK2: mid-gestational lethality caused by abnormal liver development<sup>40</sup>. The same phenotype is caused by complete loss of c-Jun<sup>42</sup>.

Although JNK1 and JNK2 are widely expressed, much attention has been paid to their functions in T cells. Unfortunately, owing to different experimental protocols and genetic backgrounds, the results of these MKK4 knockout experiments are confusing. In T thymocytes, JNK activity is synergistically stimulated by occupancy of the T-cell receptor and the CD28 auxiliary receptor and this activation parallels IL-2 induction<sup>42</sup>. Induction of anergy, which prevents T-cell activation, interferes with JNK activation<sup>43</sup>. Congruently, two groups found defective IL-2 production and proliferation after co-stimulation in JNK1/2- or MKK4-deficient T cells<sup>44,45</sup>.

This defect was also observed in *Jnk1*<sup>+/-</sup>*Jnk2*<sup>+/-</sup> double heterozygote T cells<sup>44</sup>, suggesting that partially reduced JNK1/2 activity—which is expected to occur in *Mkk4*<sup>-/-</sup> cells—is sufficient to decrease T-cell activation. At any rate, this defect is partial and bypassed by strong stimulation or antigen-presenting cells<sup>44</sup>; therefore, it is possible that neither JNK1 nor JNK2 has a major role in controlling T-cell proliferation *in vivo*. Unexpectedly, JNK1/2-deficient T cells exhibit normal AP-1 activity, but display a partial defect in activation of NF-AT, another transcription factor required for IL-2 induction<sup>45</sup>.

**Table 1 Phenotypes of MAPKK and MAPK knockout mice**

MAPKK/MAPK	Phenotypes	Similar to
MEK1	Defective placental vascularization <sup>37</sup>	ERK2?
MKK4 MKK7	Defective liver development <sup>40</sup> Embryonic lethality of unknown cause <sup>48</sup>	c-Jun knockout <sup>41</sup>
MKK3	Defective IL-12 production <sup>52</sup>	
ERK1	Defective T-cell development (positive selection) <sup>35</sup>	MEK1 dn negative transgenics
JNK1	Defective T-cell differentiation to Th2 cells <sup>46</sup>	
JNK2	Defective T-cell differentiation to Th1 cells <sup>47</sup>	
JNK1 or JNK2	Defective T-cell proliferation and IL-2 production <sup>25</sup>	JNK1 dn negative transgenics MKK4 knockouts
JNK1 or JNK2	Defective activation induced death of thymocytes <sup>25</sup>	JNK1 dn negative transgenics
JNK1 & JNK2	IL-2 overproduction <sup>48</sup>	MKK7 knockout
JNK1 & JNK2	Neural tube disclosure <sup>38,39</sup>	
JNK3	Resistance to excitotoxic neuronal cell death <sup>28</sup>	c-Jun <sup>Δ63/73</sup> knockin <sup>29</sup>
p38α	Placental defect <sup>51</sup> (trophoblast cells)	
p38α	Insufficient production of erythropoietin <sup>50</sup>	

dn, dominant-negative

Another group, however, found that *Jnk1*<sup>-/-</sup> or *Jnk2*<sup>-/-</sup> T cells produce IL-2 normally but undergo aberrant differentiation to effector cells<sup>46,47</sup>. Furthermore, an experiment in which *Jnk1*<sup>-/-</sup>*Jnk2*<sup>-/-</sup> double knockout ES cells were used to reconstitute the lymphoid lineage of immunodeficient mice suggests that JNK1/2 negatively regulated IL-2 production<sup>48</sup>.

These results are supported by analysis of *Mkk7*<sup>-/-</sup> T cells, which exhibit defective JNK activation<sup>48</sup>, but they contradict experiments in which inhibition of JNK activity correlates with decreased IL-2 expression<sup>42,43</sup>. Although this discrepancy is not fully explainable, each experiment relied on a different approach and *Mkk7*<sup>-/-</sup> T cells also exhibit much higher p38 activity than wild-type cells<sup>48</sup>. It is therefore worthwhile to evaluate whether the overproduction of IL-2 is sensitive to p38 inhibitors.

Nevertheless, instead of T-cell activation JNK1 and JNK2 may be more intimately involved in formation of effector T cells<sup>46,47</sup>. As T cells lacking both JNK1 and JNK2 differentiate along the T-helper cell type-2 (Th2) path<sup>48</sup>, the most important function of JNK1/2 in T cells could be to direct non-differentiated cells to the Th1 fate. JNK1/2 may also be involved in negative selection of T cells<sup>25,44</sup>. JNK-deficient cells, however, remain sensitive to other inducers of apoptosis, providing another example of the cell type- and stimulus-specific involvement of JNK in apoptosis. Surprisingly, no B-cell defects were detected in any of the JNK-deficient mice<sup>25,44,46–48</sup>, but a partial proliferation defect was found in *Mkk4*<sup>-/-</sup> B cells<sup>49</sup>.

In contrast to JNK1/2, the JNK3 isoform is expressed predominantly in the nervous system. *Jnk3*<sup>-/-</sup> mice are resistant to excitotoxic killing of neurons in the CA1 layer of the hippocampus<sup>28</sup>. The same phenotype is exhibited by c-Jun<sup>Δ63/73</sup> mice<sup>29</sup>. Thus, in this apoptotic pathway as in ultraviolet-irradiated fibroblasts<sup>32</sup>, the function of JNK is exerted through its effects on gene expression.

p38. The only p38 isozyme whose *in vivo* function has been examined genetically is p38α. Unlike deletions of individual *Jnk* loci or *Erk1*, inactivation of p38α results in embryonic lethality<sup>50,51</sup>. It is not clear whether the lack of compensation by other isoforms is indicative of distinct biochemical functions or a marked difference in expression patterns. The severity of the phenotype and the cause of death vary with the genetic background in which the p38α deficiency is examined. In an inbred C57Bl6/J background, p38α deficiency results in lethality before day 11 of gestation, owing to defective placental development<sup>51</sup>. However, in a mixed background, some p38α<sup>-/-</sup> mice survive longer<sup>50</sup>.

Analysis of these mice suggests that after passing the earlier hurdle imposed by development of placental trophoblasts; p38α-deficient



mice face a second hurdle owing to insufficient production of erythropoietin (Epo). Experiments with human hepatoma cells—a surrogate for fetal liver, the main site of fetal Epo production—indicate that p38 $\alpha$  is activated in response to hypoxia and that its activation is required for hypoxia-induced stabilization of *Epo* mRNA<sup>50</sup>. Regardless of the molecular processes in which p38 $\alpha$  participates, these experiments show that a MAPK previously thought to be mostly involved in inflammation and stress does have critical developmental functions.

Consistent with the presence of two MAPKKs that activate p38 MAPKs, *Mkk3*<sup>-/-</sup> mice are viable without obvious abnormalities<sup>52</sup>. Although *Mkk3*<sup>-/-</sup> macrophages exhibit reduced endotoxin-induced p38 activity, most cytokines in these cells are normally induced following endotoxin exposure with the exception of IL-12 p35 and p40 subunits<sup>52</sup>. Although this defect occurs at the transcriptional level, the p38 responsive transcription factor is unknown.

## Conclusions and prospects

In mammals, MAPK signalling cascades regulate important cellular processes including gene expression, cell proliferation, cell survival and death, and cell motility. With a few exceptions (mostly transcription factors), the MAPK substrates that regulate these processes are not known and their identification is a major challenge. The definition of physiologically relevant MAPK substrates should result in a thorough understanding of MAPK action. Currently, the only process in which a target (c-Jun) for a MAPK (JNK3) has been genetically defined is excitotoxic neuronal apoptosis<sup>28,29</sup>. As revealed by the involvement of c-Jun, this response is mediated through an effect on gene expression.

Similar analysis can be used to determine how many other biological responses to MAPK activation are mediated through changes in gene expression. As different cells express distinct sets of transcription factors, transcriptional control can easily account for much of the cell-type specificity in MAPK action. Techniques such as DNA microarray analysis should enable the definition of genes that are regulated in response to individual MAPKs, whereas proteomic analyses can be applied to the identification of substrates. Finally, specific inhibitors of MAPK signalling are becoming available. In addition to their utility in identifying MAPK functions, it is likely that they will prove useful in treating conditions, such as inflammation or cancer, caused by deregulation of MAPK cascades. □

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## Acknowledgements

We thank M. Cobb and E. Wagner for suggestions and criticism. M.K. is an American Cancer Society Research Professor; work in his lab is supported by the NIH and State of California Cancer Research Program. L.C. is supported by a NIH postdoctoral fellowship. Our apologies to those whose work was not cited due to space limitations.

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# Stress signaling in *Drosophila*

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Cells commonly use multiprotein kinase cascades to signal information from the cell membrane to the nucleus. Several conserved signaling pathways related to the mitogen activated protein kinase (MAPK) pathway allow cells to respond to normal developmental signals as well as signals produced under stressful conditions. Genetic and molecular studies in *Drosophila melanogaster* over the last several years have related that components of stress signaling pathways, namely the Jun kinase (JNK) and p38 kinase signaling modules, are functionally conserved and participate in numerous processes during normal development. Specifically, the JNK pathway is required for morphogenetic movements in embryogenesis and generation of tissue polarity in the adult. The role of the p38 pathway in generation of axial polarity during oogenesis has been inferred from phenotypic analysis of mutations in the *Drosophila* homolog of DMKK3. In addition to their requirement for normal development, cell culture and genetic investigations point to a role for both the JNK and p38 pathways in regulation of the immune response in the fly. This review details the known components of stress signaling pathways in *Drosophila* and recent insights into how these pathways are used and regulated during development and homeostasis.

**Keywords:** dorsal closure; morphogenesis; tissue polarity; immunity; JNK; p38

## Introduction

Eukaryotic cells detect and respond to a variety of external signals and stresses by utilizing evolutionarily conserved protein kinase signaling modules, such as the mitogen-activated protein kinase (MAPK) cascades. These multiprotein modules afford versatility to cells at several levels. First, a cascade of consecutive kinases allows for signal amplification and multiple sites of regulation. Second, the kinase module can couple to diverse receptors for signal detection and integration. Finally, interactions with different effectors can elicit a diverse array of cellular responses.

Reflecting the large variety of signals that cells encounter, numerous related but functionally distinct MAPK cascades have evolved (Widmann *et al.*, 1999) (Table 1). Signals from growth factors or mitogens are typically transduced by the MAPK/ERK (extracellular signal-responsive kinase) signaling pathway. Two other related pathways are activated in response to stress stimuli, such as mechanical deformation, UV irradiation and heat shock, as well as to hormones, and cytokine

ligands (Kyriakis, 1999). These stimuli signal activation of the stress-activated protein kinases (SAPKs), exemplified by the Jun amino (N)-terminal kinase (JNK), and the p38 kinases. Like MAPK/ERK kinases, JNK and p38 catalytic activity is stimulated in response to stress signals by phosphorylation and activation of several upstream kinases, JNK kinase (JNKK or MKK) and JNK kinase kinase (JNKKK, MKKK or MEKK). Kinase cascades of this sort are typically linked to regulation of gene expression, because transcription factors are often the targets of regulatory phosphorylation events. In the stress response pathway, one target of JNK phosphorylation is the c-Jun transcription factor, that mediates transcriptional regulation of gene expression when in complex with c-Fos to form the activator protein-1 (AP-1) transcriptional regulator; ATF2 (activated transcription factor-2) is another common substrate of both the JNK and p38 pathways (Kyriakis and Avruch, 1996; Paul *et al.*, 1997).

Stress response outputs in mammalian cells mediated by the JNK and p38 pathways are equally as diverse as the inputs and include cell cycle regulation, inflammatory response, cell growth, differentiation, and apoptosis (Kyriakis and Avruch, 1996; Paul *et al.*, 1997). In unicellular yeast cells, the more distantly related MAPK cascades also regulate cell cycle, but in addition, function in the mating response, osmotic homeostasis and cell wall remodeling (Gustin *et al.*, 1998; Herskowitz, 1995; Toone and Jones, 1998). Common features of these diverse behavioral responses from yeast to man involve not only transcriptional regulation of gene expression but also reorganization of cytoskeletal elements and thus cell shape, adhesion, and orientation. It is, therefore, becoming increasingly important to recognize and understand the role of regulatory interplay between the cellular signal transduction machinery and the cellular structural machinery. In fact, accumulating evidence suggests that specificity in signaling can be achieved by spatially restricting activation of signaling components through interactions with scaffolding proteins localized to the cytoskeleton or membrane (Garrington and Johnson, 1999; Madhani and Fink, 1998).

Components of all three prototypical MAPK cascades have now been identified and characterized in *Drosophila melanogaster*. Genetic studies in the fly emphasize the versatility of MAPK signal transduction pathways for normal developmental processes. In the past several years in particular, we have witnessed a bounty of new findings about the regulation and function of stress signaling pathways. Mutations in the *Drosophila* homologs of JNK pathway components result in a common embryonic phenotype implicating their combined function in the morphogenetic process of dorsal closure (DC) during embryogenesis. Further

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Table 1 Evolutionarily conserved MAPK signaling pathways

	Yeast mating response		Mammals		Fly dorsal closure
Input	Mating pheromone	Mitogens	Stress, inflammatory cytokines		?
MEKKK	Ste20		PAK	NIK, GCK, HPK	Men
MEKK (MKKK)	Ste11	Raf	ASK, TAK	MEKK1,4	?
MEK (MKK)	Ste7	MEK1,2	MKK3,6	MKK4,7	Hep
MAPK	Fus3	ERK1,2	p38	JNK	Bek
Target	Far1	Elk1	ATF2	AP-1	AP-1

analysis has revealed additional requirements for several components of the JNK pathway in immunity and the generation of cellular polarity. Components of the p38 pathway have also been identified, but to date, the phenotypic consequences of mutations in the genes have only been reported for *DMKK3/licorne* defining its role in establishment of axial polarity during oogenesis. Though mutations in the two p38 kinases have not yet been characterized, genetic, biochemical, and cell culture studies have set the stage for characterization of p38 kinase cascades in immunity and morphogenesis.

The focus of this review is an update on stress signaling cascades in *Drosophila*. The roles of the JNK and p38 pathways are being defined largely in the context of normal development. Though we know surprisingly little about the molecular nature of cellular responses to physiological stresses on the fly in its natural environment, it is likely that the stress response involves inducible activity of the JNK and p38 cascades. Investigation of JNK and p38 activity during an immune challenge may shed light on the regulation of this inducible response. In this review, we detail recent observations about newly identified components within these two pathways and discuss their utilization during development and homeostasis. Several recent reports have brought new insights as well as new questions to the table with respect to how JNK pathway activity is regulated and how it may interface with the cytoskeleton.

#### The JNK signaling pathway is required for morphogenesis in *Drosophila*

Elements of the stress signaling pathway, specifically DJun, JNK, and JNKK, have been cloned and characterized in *Drosophila* over the last several years (Glise *et al.*, 1995; Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996). These investigations have shown not only that the genes are present in the fly and highly conserved at the sequence level in comparison to their mammalian counterparts, but also that they function in a concerted signal transduction pathway to regulate gene expression and cytoskeletal remodeling during embryogenesis. In particular, mutations in JNK pathway components disrupt a morphogenetic process referred to as dorsal closure (DC).

DC is the movement of the dorsolateral epidermis on either side of the embryo toward the dorsal midline to cover a degenerative squamous epithelium, the amnioserosa, and to enclose the embryo in a continuous

epithelium (Figure 1). Closure relies predominantly on cell shape changes within the postmitotic epidermis, instead of cell division or cell intercalation (Campos-Ortega and Hartenstein, 1985; Young *et al.*, 1993). Initially, just after retraction of the embryonic germband is complete, cells at the leading edge (LE) of the dorsal epidermis, which abut the amnioserosa, begin to elongate dorsally. At the dorsalmost membrane of these cells adjacent to the amnioserosa, there is an obvious enrichment of cytoskeletal proteins such as nonmuscle myosin and actin (Young *et al.*, 1993). Concomitant with cytoskeletal remodeling and changes in cell shape from polygonal to elongated, LE cells upregulate expression of at least two genes including *decapentaplegic (dpp)*, a TGF- $\beta$ -like molecule, and *puckered (puc)*, a phosphatase (Ring and Martinez-Arias, 1993; St. Johnston and Gelbart, 1987). After the initial elongation of LE cells, a spreading phase ensues. LE cells become markedly more narrow and stretched, presumably due to contraction of the actin-myosin network, resulting in

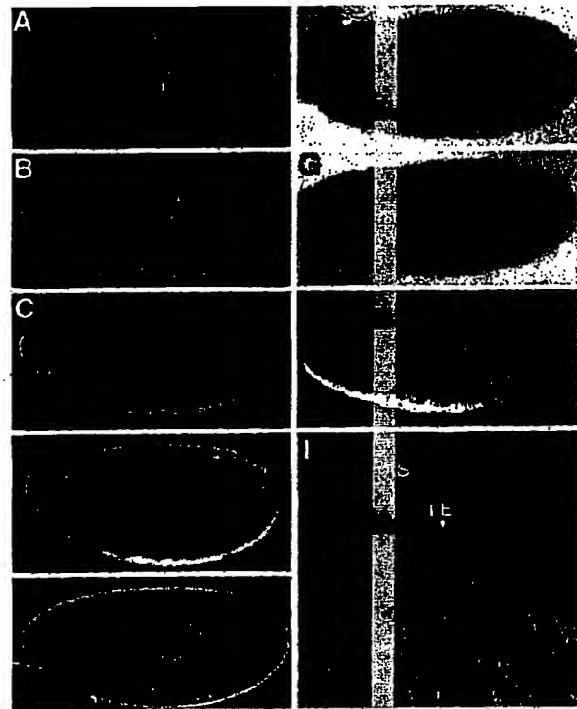


Figure 1 Movements, phenotypes, molecular markers, and cell shape changes associated with dorsal closure in *Drosophila*. Scanning electron micrographs of embryos reveal the morphogenetic movements of dorsal closure (a-e). Just after germband retraction (a), the leading edge, LE, of the epidermis begins to move dorsally toward the midline to cover a degenerative squamous epithelium, the amnioserosa, AS (b). At the end of embryogenesis, the embryo is completely surrounded by epidermis (c) which secretes a continuous patterned cuticle (d). Mutant embryos in which dorsal closure fails, produce cuticles with dorsal holes (e). During closure, JNK pathway signaling results in the expression of two transcriptional targets in LE cells, *dpp* and *puc* (see text for details). *dpp* transcripts are observed in several tissues including the LE during germband retraction (f) and dorsal closure (g). The phosphatase, *Puc*, is detected by expression of a *LacZ* transgene inserted in the *puc* locus. *Puc* expression is restricted to LE cells, shown here late in dorsal closure when they meet at the dorsal midline (h). Immunofluorescent labeling of cell membranes in the epidermis show the dramatic stretching of LE and lateral cells during closure (i). In all panels, anterior is to the left and dorsal is up.



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the dorsalward movement of the whole epithelium toward the midline (Young *et al.*, 1993). Also during this phase, a wave of cellular elongation proceeds ventrally away from the LE to include more lateral cells. These dramatic shape changes are eventually sufficient to allow the entire dorsolateral epithelium to cover the region previously occupied by the amnioserosa (Young *et al.*, 1993). DC ends with suturing or fusion, in which the epithelia on both sides of the embryo finally meet at the dorsal midline and adhere to one another without continuing to move over the midline. The entire process takes approximately 2 h to complete.

Embryos that fail to complete DC because of mutations in genes required for the process exhibit a characteristic dorsal hole in the cuticles they secrete (Figure 1). This is due to the fact that amnioserosa cells normally degenerate when enclosed inside the epidermis and do not contribute to the cuticle (Campos-Ortega and Hartenstein, 1985). Therefore, if amnioserosa cells are left exposed in embryos which fail to close, the cuticle is not complete. The 'dorsal open' cuticle has been used successfully in genetic screens as a criterion for isolating genes which function during the process of DC (Chou and Perrimon, 1996; Nusslein-Volhard *et al.*, 1984; Perrimon *et al.*, 1989). In this way, more than two dozen genes have been characterized that when mutant either zygotically, or maternally, or both, give rise to varying degrees of open cuticles. Most of the genes fall into several groups based on the proposed functions of each gene product

(Table 2). For instance, one class of dorsal open mutants define genes encoding components of the cytoskeleton or cell adhesion machinery. A second group of genes implicated in DC are members of the JNK signal transduction pathway. A third group is represented by genes encoding components of the Dpp signal transduction pathway. Finally, additional molecules are thought to participate in DC based not on mutant phenotype but on overexpression studies or suggestive expression patterns. Most notable are the small GTPases of the Ras superfamily, Rho, Rac and Cdc42 (among which *RhoA* mutants have been reported) and their associated proteins such as DPAK, the *Drosophila* homolog of p21-activated kinase (Harden *et al.*, 1996, 1999; Strutt *et al.*, 1997).

Extensive genetic epistasis and interaction studies coupled with biochemical and molecular data have led to an integrated model describing the regulatory control and cellular mechanisms driving DC (Figure 2). One of the first events is thought to be activation of the JNK signaling pathway in the LE cells. Members of the core JNK signaling module that have been cloned in *Drosophila* include *hemipterous* (*hep*), a JNKK and functional homolog of mammalian MKK7, *basket* (*bsk*), a JNK, and *DJun* (Glise *et al.*, 1995; Holland *et al.*, 1997; Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996). Mutations in each gene lead to embryos with severe dorsal and anterior open cuticles indicative of a failure of DC and head involution. Loss of JNK pathway activity has

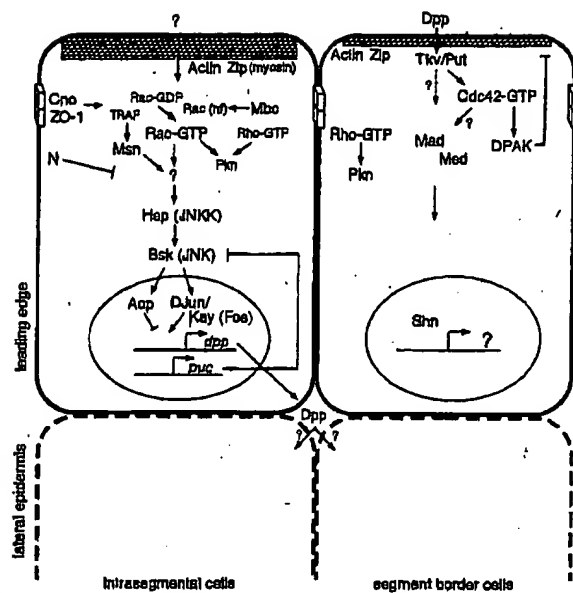
Table 2 Genes involved in DC

Gene	Protein structure	Reference
<b>Cytoskeleton/adhesion</b>		
<i>myospheroid</i>	Integrin $\beta$ subunit	(Brown, 1994)
<i>scab</i>	Integrin $\alpha$ subunit	(Stark <i>et al.</i> , 1997)
<i>coracle</i>	Band 4.1	(Fehon <i>et al.</i> , 1994)
<i>zipper</i>	Nonmuscle myosin heavy chain	(Young <i>et al.</i> , 1993)
<i>collagen</i>	Extracellular matrix	(Borchellini <i>et al.</i> , 1996)
<i>canoe</i>	PDZ protein	(Jurgens <i>et al.</i> , 1984; Takahashi <i>et al.</i> , 1998)
<i>ZO-1</i>	PDZ and guanylate kinase	(Takahashi <i>et al.</i> , 1998)
<i>discs large</i>	PDZ and guanylate kinase	(Perrimon, 1988)
<i>Notch</i>	Transmembrane receptor	(Zecchini <i>et al.</i> , 1999)
<i>raw</i>	Unknown	(Blake <i>et al.</i> , 1998)
<i>ribbon</i>	Unknown	(Blake <i>et al.</i> , 1998)
<b>GTPases and effectors</b>		
<i>Drac1</i>	GTPase	(Glise & Noselli, 1997; Harden <i>et al.</i> , 1995; 1999)
<i>Cdc42</i>	GTPase	(Glise & Noselli, 1997; Harden <i>et al.</i> , 1995; 1999)
<i>DRho1</i>	GTPase	(Harden <i>et al.</i> , 1999; Strutt <i>et al.</i> , 1997)
<i>Pkn</i>	Protein kinase C-related kinase	(Lu and Settleman, 1999)
<i>myoblast city</i>	DOCK180/cdc-5 homolog	(Erickson <i>et al.</i> , 1997; Nolan <i>et al.</i> , 1998)
<i>DPAK</i>	p21-associated kinase	(Harden <i>et al.</i> , 1996)
<b>JNK pathway</b>		
<i>misshapen</i>	Ste20 family kinase	(Su <i>et al.</i> , 1998)
<i>hemipterous</i>	Jun N-terminal kinase kinase	(Glise <i>et al.</i> , 1995)
<i>basket</i>	Jun N-terminal kinase	(Riesgo-Escovar <i>et al.</i> , 1996; Sluss <i>et al.</i> , 1996)
<i>DJun</i>	Jun transcription factor	(Glise and Noselli, 1997; Hou <i>et al.</i> , 1997; Kockel <i>et al.</i> , 1997; Riesgo-Escovar and Hafen, 1997)
<i>kayak</i>	Fos transcription factor	(Zeitlinger <i>et al.</i> , 1997)
<i>anterior open/yan</i>	ETS domain	(Lai and Rubin, 1992; Riesgo-Escovar and Hafen, 1997)
<i>puckered</i>	VH-1 family phosphatase	(Martin-Blanco <i>et al.</i> , 1998; Ring and Martinez-Arias, 1993)
<b>DPP pathway</b>		
<i>decapentaplegic</i>	Transforming growth factor $\beta$	(Padgett <i>et al.</i> , 1987; St. Johnston and Gelbart, 1987)
<i>thick veins</i>	TGF $\beta$ type I receptor	(Affolter <i>et al.</i> , 1994; Brummel <i>et al.</i> , 1994; Nellen <i>et al.</i> , 1994; Penton <i>et al.</i> , 1994)
<i>punt</i>	TGF $\beta$ type II receptor	(Lettsou <i>et al.</i> , 1995; Ruberte <i>et al.</i> , 1995)
<i>mothers against dpp</i>	R-smad	(Hudson <i>et al.</i> , 1998)
<i>medea</i>	Co-smad	(Das <i>et al.</i> , 1998; Hudson <i>et al.</i> , 1998; Wisotzkey <i>et al.</i> , 1998)
<i>schnurri</i>	Nuclear zinc-finger	(Arora <i>et al.</i> , 1995; Grieder <i>et al.</i> , 1995; Staehling-Hampton <i>et al.</i> , 1995)

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**Figure 2** The JNK pathway signaling cascade regulates dorsal closure. This model takes into consideration many observations reported over the last few years, however, it is not inclusive of every molecule that has been described to play a role in dorsal closure. Prior to dorsal closure, the JNK pathway is activated by an unknown mechanism in leading edge cells via the action of the GTPase, Rac. Many proteins contribute to regulation of the JNK pathway including components of cell junctions, Cno and ZO-1, a Rac-interacting protein, Mbc and a ste20 family member, Msn, all of which can exert a positive effect on signaling. Membrane-associated N appears to negatively regulate the JNK cascade. Signaling through the Rac/JNK pathway leads to accumulation of an actomyosin cytoskeletal network at the dorsal membrane of LE cells and also activates the Jun/Fos AP-1 transcription factor resulting in upregulation of two transcriptional targets, *dpp* and *puc*. Puc, a phosphatase for Bsk, participates in negative feedback regulation of JNK signaling in the leading edge. Dpp is secreted and stimulates a conserved signaling pathway via the receptors, Tkv and Put, and the effectors, Med and DPAK. Recent data supports the view that Dpp signal transduction predominates in the LE, at or near segment borders, however Dpp may still signal to the lateral epidermis to mediate the spread of cell shape changes more ventrally. Dpp signaling appears to require the GTPase Cdc42, and through its effector, DPAK, regulates the extent of cytoskeletal accumulation along the LE and thus mechanics of closure across the epidermis. The GTPase, Rho, and its effector kinase Pkn, have also been implicated in closure; this pathway may be restricted to a subpopulation of cells in the LE. Refer to text for additional details

several consequences that contribute to a failure of DC. Mutant embryos exhibit a loss of *dpp* expression in LE cells without perturbing other elements of the *dpp* expression pattern (Glise and Noselli, 1997; Hou *et al.*, 1997; Riesgo-Escovar and Hafen, 1997). *puc* expression is similarly affected. JNK pathway mutants also fail to accumulate the proper cytoskeletal network in LE cells and thus LE cells do not fully elongate dorsally (Hou *et al.*, 1997; Ricos *et al.*, 1999). Lateral cells never elongate when the JNK signaling cascade is blocked by mutation suggesting that Dpp, a JNK pathway transcriptional target and secreted factor, may mediate the ventrally directed wave of cellular elongation observed in wild-type embryos during DC. LE expression of a constitutively activated form of c-Jun, *Jun<sup>act</sup>*, is capable of rescuing not only the cuticle phenotype caused by mutations in either *bsk* or *hep* but also of restoring *dpp* expression at the LE and cell elongation in the lateral epidermis (Hou *et al.*, 1997; Riesgo-Escovar and

Hafen, 1997). These results emphasize several important facets of the JNK signaling cascade and the DC system in general. First, signaling through the JNK pathway is required only in the LE and results in activation of the Jun/Fos AP-1 complex. Second, a critical outcome of signaling via Jun/Fos appears to be transcriptional upregulation of *dpp* in the LE and subsequent signaling via the Dpp pathway to cells in more lateral positions in the epithelium. A prediction of this model linking the two signaling pathways is that restoration of Dpp signaling should alleviate the block to DC caused by mutations in positively acting components of the JNK pathway. Indeed, targeted expression in lateral epidermal cells of an activated Dpp receptor, *tkv<sup>act</sup>*, rescues partially the loss of *bsk* or *DDJun* function presumably by mediating the effects of Dpp in the lateral cells (Hou *et al.*, 1997; Riesgo-Escovar and Hafen, 1997). Taken together with biochemical experiments demonstrating the catalytic activity and substrate specificity of Hep (JNKK) and Bsk (JNK) (Glise *et al.*, 1995; Sluss *et al.*, 1996), these results confirm that the components of the JNK signaling cascade in *Drosophila* functionally resemble the stress signaling kinase cascades defined in yeast and vertebrates.

Using similar criteria, it has been possible to establish the position of the Rac and Cdc42 GTPases within the signaling hierarchy. Dominant negative versions of Rac and Cdc42, when expressed in the dorsolateral epidermis, can block DC, *dpp* transcript accumulation, and enrichment of cytoskeletal components at the LE (Harden *et al.*, 1995; Hou *et al.*, 1997). On the other hand, ectopic expression of gain-of-function or activating mutants of Rac and Cdc42 provoke a strong ectopic upregulation of *dpp*, consistent with a positive role in regulating DC and a hyperactivation of the pathway (Glise and Noselli, 1997). These effects are suppressed in embryos additionally mutant for *hep* (Glise and Noselli, 1997). Together, these observations place Rac and Cdc42 function upstream of the JNK cassette consistent with the ability of vertebrate Rho family members to activate the JNK pathway (Coso *et al.*, 1995; Minden *et al.*, 1995).

Several negative regulators of DC have also been identified genetically. *puc* encodes a dual-specificity protein phosphatase of the VH-1 family of MAP kinase phosphatases (MKPs), that is localized specifically in LE cells (Martin-Blanco *et al.*, 1998; Ring and Martinez-Arias, 1993). Puc functions to moderate signaling through the JNK pathway by antagonizing the kinase activity of Bsk. In fact *puc* loss-of-function mutant embryo lysates have increased JNK activity in kinase assays, and enhanced *dpp* expression in embryos (Martin-Blanco *et al.*, 1998). In contrast, ubiquitous expression in embryos of wildtype Puc leads to dorsal cuticle holes and a loss of *dpp* expression indicating a substantial downregulation of JNK pathway activity (Martin-Blanco *et al.*, 1998). Therefore, Puc serves a key regulatory role in JNK signaling as a transcriptional target of the pathway that builds up and feeds back negatively on the activity of Bsk to moderate signaling through the pathway. Another negative component is encoded by the *anterior open/yan* (*aop/yan*) gene (Lai and Rubin, 1992). Whereas an activated form of Jun rescues the phenotypes associated with mutations in upstream components, *aop* loss-of-function mutations dominantly suppress *bsk* mutations (Riesgo-Escovar



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and Hafen, 1997). *aop* codes for a nuclear ETS domain protein that acts downstream of *hep* and *bsk* as a transcriptional repressor (Lai and Rubin, 1992; Riesgo-Escovar and Hafen, 1997). Phosphorylation by the Bsk kinase of Aop and DJun has the net effect of strong *dpp* expression because repression by Aop is relieved and activation by DJun is promoted.

The intricate nature of morphogenetic movements that take place during DC is reflected in the underlying molecular complexity given the large number of genes that have been identified to act in the process. Although much progress has been made in defining a fundamental signaling mechanism linking the JNK cascade to Dpp signaling to coordinate epithelial movement, additional components are still undefined. It remains unclear how the LE is determined and organized, how the JNK pathway is activated initially in these cells, and what role Dpp signaling plays in the lateral epidermis.

#### New components and complex regulation of JNK signaling during DC: the role of the GTPases

Although the dorsal open cuticle phenotype has allowed identification of many genes that participate in DC, the cuticle itself may be a rather crude indicator of the specific requirements of gene products during closure. Recent studies to elucidate the individual role of each GTPase, Rho, Rac, and Cdc42 in DC are beginning to suggest that not all cells of the LE are created equal. Even though expression of dominant negative versions of all three GTPases can result in cuticles with holes, distinct phenotypes are observed at the cellular and molecular level (Harden *et al.*, 1999; Ricos *et al.*, 1999). For instance, segmental or ubiquitous expression of dominant negative (DN)-Rho, DN-Cdc42, and DN-Rac can cause varying degrees of dorsal openness. However, while ubiquitous expression of DN-Rac and to a lesser extent DN-Cdc42 results in loss of cytoskeletal elements (actin, myosin, phosphotyrosine) in all LE cells, expression of DN-Rho leads to disruptions of LE cytoskeleton only in cells at the segment border (Harden *et al.*, 1999). Additionally, there appears to be a more robust effect of Cdc42, than the other GTPases, on the levels and activity of a presumed effector, DPAK (p21-activated kinase) (Harden *et al.*, 1999). Taking into account the presence or absence of LE cytoskeleton and the morphology of the epithelium, Ricos and colleagues have extended the analysis and show that DN-Rac shares phenotypes with JNK pathway mutants while DN-Cdc42-mediated effects more closely resemble the phenotypes associated with Dpp signaling components (Ricos *et al.*, 1999). These observations point out that there are likely to be differential GTPase requirements across the row of LE cells and uncovers for the first time in this system differences between segment border and intrasegmental cells. Utilization of the different GTPases in distinct subpopulations of LE cells may be a way to generate distinct cytoskeletal configurations; or to regulate differential adhesiveness, contractility, or signaling activities. In turn, cell type specific GTPase effectors may be required to coordinate movement of the segmented epithelium as a whole to ensure proper meeting of the contralateral cells at the midline. It will

be interesting to see if any newly discovered genes required for DC show a segmental expression pattern.

#### GTPase modulators, Mbc and Pkn

In the last year, several new regulators and effectors specific to certain GTPases have been reported and thus serve as candidates to mediate the unique GTPase functions. One modulator of Rac function was identified in a search for dominant suppressors of a Rac-induced rough-eye phenotype (Nolan *et al.*, 1998). This suppressor, *Su(rac)1*, is allelic to *myoblast city* (*mbc*), a gene previously isolated for its role in myoblast fusion during muscle development (Rushton *et al.*, 1995). *mbc* mutant embryos are defective not only in myoblast fusion, but in DC and nervous system wiring (Erickson *et al.*, 1997; Nolan *et al.*, 1998). These phenotypes are reminiscent of those associated with overexpression of activated or antimorphic forms of Rac, and together with the genetic suppression data suggest involvement of Mbc in a common pathway with Rac (Erickson *et al.*, 1997; Luo *et al.*, 1994; Nolan *et al.*, 1998). Mutations in *mbc* suppress both Rac- and Cdc42-, but not Rho-induced eye defects (Nolan *et al.*, 1998). However, in cotransfection assays in vertebrate cells, the human homolog of Mbc, DOCK180, interacts exclusively with Rac in a nucleotide-independent manner (Nolan *et al.*, 1998). Human DOCK180 has been implicated in regulating cell morphology when targeted to the cell membrane (Hasegawa *et al.*, 1996). In addition, biochemical data revealed a physical interaction between DOCK180 and Crk, an adaptor protein localized to sites of cell adhesion (Hasegawa *et al.*, 1996). Taken together, a plausible functional model consistent with the reported data is that Mbc/DOCK180 may influence Rac-mediated changes in membrane morphology or cytoskeleton through recruitment to specific subcellular sites or through localized activation of Rac at the membrane.

What is the role, if any, of Mbc in Rac-dependent transcriptional control? Although activated Rac and DOCK180 can both stimulate JNK activity in transfected cells as measured by an increase in Jun phosphorylation, *mbc* mutant embryos show limited effects on *dpp* expression in the LE (Nolan *et al.*, 1998). These results raise the possibilities that there are alternative ways to activate Rac and thus the JNK pathway during DC or that Rac may not be the sole input to activate the JNK pathway. Furthermore, Rac may participate in some LE functions independent of its role in transcriptional control. Epistasis analysis revealed that DN-Rac cotransfected with DOCK180 could inhibit the DOCK180-dependent stimulation of JNK catalytic activity, placing Mbc/DOCK180 upstream of Rac (Nolan *et al.*, 1998). In conclusion, Mbc appears to be a specific upstream modulator of Rac function in several developmental processes that require extensive cytoskeletal remodeling and changes in membrane morphology. Questions still remain over the exact nature of Rac regulation by Mbc and whether *mbc* mutant defects are mediated solely through the cytoskeleton or via transcriptional control using the JNK signaling pathway.

Another GTPase-interacting protein, *Drosophila* protein kinase N (Pkn), has been implicated in DC

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(Lu and Settleman, 1999). Pkn is a member of the protein kinase C-related serine/threonine kinases and is the predominant kinase associated with Rho in mammalian tissues (Vincent and Settleman, 1997). In tissue culture cells, transfected *Drosophila* Pkn specifically associates with the activated GTP-bound forms of Rho and Rac in pull-down assays and its catalytic activity is enhanced slightly in response to these interactions (Lu and Settleman, 1999). *pkn* mutant embryos have a nearly identical dorsal open cuticle phenotype as embryos zygotically mutant for *rhoA*; however, *pkn* does not suppress Rho- or Rac-induced defects in the eye or perturb any gastrulation processes for which Rho has been implicated. Therefore, Pkn appears to be a specific GTPase effector for DC. Interestingly, similar to *mbc* mutants and embryos expressing DN-Rho, but in contrast to JNK pathway components, *pkn* mutations do not significantly perturb *dpp* expression in the LE (Lu and Settleman, 1999). This is one of the first GTPase effectors that does not seem to influence JNK transcriptional targets during DC, while still producing a strong cuticle phenotype and defects in the LE cytoskeleton. Thus, Yu and Settleman (1999) propose that Pkn acts predominantly in a Rho-specific pathway in parallel with the Rac-JNK pathway to control LE behavior and cytoskeletal remodeling, though their data does not rule out the possibility that Pkn may mediate some Rac functions independent of the downstream JNK cascade. Nonetheless, it is likely that the two pathways, Rho-Pkn and Rac-JNK, eventually converge on some common function because both are clearly required for LE cell stretching and ultimately DC. It is formally possible, however, that the two pathways literally function independently because they are restricted to distinct cell types within the LE. Finally, characterization of *pkn* mutants begs the question of the role of Dpp in lateral cell elongation because lateral cells do not elongate in *pkn* mutants even though *dpp* is still expressed. One explanation is that the Rho-Pkn pathway generates a second signal required for lateral cell stretching that acts coordinately with Dpp. Alternatively the function of the Dpp signaling pathway may not be to promote lateral cell stretching per se but is permissive for those cell shape changes. Perhaps the answer simply involves the biomechanics of the system, that cells in the lateral epidermis cannot elongate unless or until LE cells have already done so despite the presence of all the correct signals.

#### MK4444s and TRAF

In an effort to determine the mechanism by which the JNK pathway is activated and DC is initiated, many groups have sought to identify upstream components in the pathway through characterization of new dorsal open mutants or cloning functional candidates, by analogy with signaling pathways elucidated in other organisms. These methods have successfully converged to implicate *misshapen* (*msn*) in the regulation of DC and JNK signaling (Su *et al.*, 1998). *Msn* was originally identified as a target of an eye-specific transcription factor and *msn* mutant clones in the eye lead to abnormal photoreceptor morphology (Treis-

man *et al.*, 1997). Embryos zygotically mutant for *msn* show a low penetrance dorsal open phenotype (Su *et al.*, 1998). Cloning the *msn* gene revealed that it is a member of the growing superfamily of protein kinases related to mammalian PAK and yeast sterile 20 (ste20), a kinase required for the mating response (Herskowitz, 1995). These kinases could be considered as MK4444s (or MEK4444s), which phosphorylate and activate MK4444s (JNK4444s or MEK4444s). Thus, by analogy *Msn* could presumably activate an as yet unknown kinase for which *Hep*, the *Drosophila* JNK4444, would be the substrate. To address whether *Msn* indeed impinges on the JNK cascade in *Drosophila*, cellular and genetic analysis was undertaken. Both *Msn* and its mammalian homolog, NIK (NCK-interacting kinase), can stimulate JNK activity in transfected cells (Su *et al.*, 1998). This activation is markedly decreased by simultaneous cotransfection of DN-Rac, suggesting that *Msn* may be upstream of Rac. Although *Msn* may lie in a signaling pathway with Rac, it does not interact directly with Rac (by two-hybrid or overlay assay), consistent with its lacking a defined GTPase binding domain (Su *et al.*, 1998). Unlike typical ste20 kinases, such as PAK, which exhibit Rac and Cdc42 binding domains coupled to C terminal kinase domains, SPS-1 subfamily ste20-related kinases, for which *Msn* is highly related, have no GTPase binding sites and have N-terminal kinase domains (Kyriakis, 1999). Thus currently, just where *Msn* fits into the JNK signaling hierarchy is still somewhat unclear but perhaps *Msn* and Rac cooperate to activate a kinase upstream of *Hep* similar to the way Ras and an as yet unidentified kinase converge to activate Raf and subsequently the MAPK cascade (Morrison and Cutler, 1997).

*In vivo* genetic analysis lends further support to the notion that *Msn* is required for signaling through the JNK pathway. For example, in *msn* mutant embryos, there is a substantial reduction in *dpp* transcript accumulation in the LE (Su *et al.*, 1998). Additionally, expression of an activated form of c-Jun or an activated Tkv receptor can partially rescue *msn* mutant embryos. To address how *Msn*, and ste20 kinases in general, are activated and regulated, *Msn* sequences were used in the yeast two-hybrid assay to identify interacting protein partners. One protein identified was *Drosophila* TRAF1 (TNF-receptor associated factor) (Liu *et al.*, 1999). Extensive analysis of TRAF family members in mammalian tissues has shown that TRAFs serve as adaptor proteins to link cell surface receptors to both the NF- $\kappa$ B pathway and the JNK pathway (Arch *et al.*, 1998). *In vitro* kinase assays demonstrate that both *Drosophila* *Msn* and TRAF1 can stimulate JNK activity in transfected cells (Liu *et al.*, 1999). Stimulation of Jun phosphorylation by TRAF1 can be blocked, however, by cotransfection of a dominant negative form of *Msn*, lacking the kinase domain, suggesting that TRAF1 is likely to be upstream of *Msn* in signaling, consistent with the placement of TRAFs in vertebrate signaling pathways (Arch *et al.*, 1998; Kyriakis, 1999; Liu *et al.*, 1999). Thus, *Msn* and perhaps TRAF1 could be the most upstream components yet identified in the regulatory signaling cascades controlling DC, but confirmation awaits a genetic analysis of TRAF1 in *Drosophila* development.





## Notch

As an old player with another new function, Notch (N) may contribute to the regulation of morphogenesis, independent of its better known nuclear pathway. Zygotic *N* mutant embryos have a neurogenic phenotype due to the failure of lateral inhibition during the specification of neural versus epidermal tissue (Artavanis-Tsakonas *et al.*, 1999). Thus, neural tissue hypertrophy on the ventral side of the embryo, at the expense of epidermis, leaves just a scrap of dorsal cuticle. Loss of both maternal and zygotic *N* function increases the severity of the cuticle phenotype such that cuticles are even further reduced in size and exhibit dorsal defects, including what appears to be a dorsal midline hole (Zecchini *et al.*, 1999). This phenotype suggests that *N* may have a function in dorsal tissue during the process of DC. To establish a potential mechanism for the effect of *N* on DC, JNK target gene expression was examined. Although the morphology of the embryos mutant for *N* is severely distorted, it appears that both *dpp* and *puc* are detected ectopically; *dpp* then fades earlier than normal (Zecchini *et al.*, 1999). Although this may be due to hyperactivation of the JNK pathway, similar to what is observed in embryos programmed to express activated Rac or in embryos mutant for negative regulators of the JNK pathway such as *Puc*, it seems feasible that the *N* effect could be due not to ectopic expression via JNK activation but by 'hypertrophy' of the LE. In other words, if *N* functions in LE cell specification at the boundary of two cell sheets, the amnioserosa and the dorsal epidermis, then the increased expression of LE specific molecules may reflect an increased number of LE cells that have differentiated due to failure in lateral inhibition. The function of *N* in lateral inhibition is mediated by an intracellular pathway involving cleavage of the *N* cytoplasmic domain, translocation to the nucleus, and transcriptional upregulation of genes in cooperation with the Suppressor of Hairless (*Su(H)*) protein (Artavanis-Tsakonas *et al.*, 1999). The effects of *N* in DC appear not to require this pathway, though, for several reasons. First, mutations in *Su(H)* do not confer dorsal defects nor changes in *dpp* expression. In addition, DC defects mediated by *N* require *N* to be at the cell surface and can be manifested in embryos expressing various *N* constructs that cannot be proteolytically processed (Zecchini *et al.*, 1999). Thus, it is unlikely that the requirement of *N* in DC works through a pathway involving lateral inhibition on the dorsal side of the embryo but rather uses a mechanism that moderates JNK activity more directly. Consistent with this notion, extracts from *N* mutant embryos show increased JNK activity and Jun phosphorylation, which can be suppressed by mutations in *hep* (Zecchini *et al.*, 1999). Thus, collectively, it appears that *N* is a new negative regulator of the JNK pathway in DC and serves to antagonize JNK activation presumably through a position at the cell membrane.

## Cell junction proteins

An emerging theme in *Drosophila* DC, as well as many other systems, is that signaling pathways are subject to regulation by cellular junctions. This is likely to be an essential way that cells limit or extend responses to

secreted factors by the integration of signals from cell-cell or cell-matrix receptors poised to monitor cell adhesion, polarity, and spatial position. It is probably at or near the membrane that this signal integration takes place. With this in mind, a recent report characterizing *cano* (*cno*), a gene previously identified as a DC mutant (Jurgens *et al.*, 1984), emphasizes the importance of cellular junctions for JNK signaling and the regulation of DC movements (Takahashi *et al.*, 1998). Genetic studies reveal an interaction between *cno* and components of the JNK pathway. Specifically, loss of one copy of *hep* or *bsk* can enhance an embryonic viable *cno* allele combination whereas in a complimentary experiment, overexpression of wildtype *bsk* can suppress or rescue a slightly stronger *cno* allele combination. Examination of JNK target genes reveals that indeed *dpp* and *puc* are significantly reduced in *cno* mutant embryos in comparison with wildtype embryos. Thus, like other positively acting components of the JNK pathway, *Cno* permits or stimulates signaling. So where might *Cno* fit into the JNK pathway? Since *cno* mutations do not block the stimulation of target genes induced by expression of activated Rac, it appears that *Cno* is required upstream of or in parallel with the GTPase, Rac. Further genetic and biochemical evidence indicates that *Cno* interacts with *Drosophila* ZO-1, whose mammalian homolog is a known component of cellular junctions and member of the MAGUK family of proteins containing protein-binding PDZ and guanylate kinase domains (Itoh *et al.*, 1993; Stevenson *et al.*, 1986). Interestingly, *cno* encodes a protein that also contains a PDZ domain and, in addition, contains motifs that resemble kinesin, myosin V, and Ras binding domains (Miyamoto *et al.*, 1995; Ponting, 1995). Examination of *Cno* protein distribution in fly embryos reveals broad tissue distribution but restricted subcellular localization to the adherens junctions of epithelial cells (Takahashi *et al.*, 1998). ZO-1 is similarly distributed but shows a broader membrane localization encompassing both adherens and septate junctions of epithelial cells. It is provocative that JNK signaling and DC are defective in *cno* and ZO-1 mutants whose wildtype functions presumably contribute to junction assembly, maintenance, or regulation and suggest that there is crosstalk between sites of cell adhesion and the proteins involved in initial activation the JNK pathway. This scenario has been demonstrated elegantly in the nematode *C. elegans*. Genetic studies have shown that vulval induction requires the proper spatial distribution of the EGF receptor, which subsequently signals through a Ras-Raf-MAPK cassette (Kim, 1995). Mislocalization of the receptor in mutants which disrupt epithelial polarity or junctional integrity or both, causes abnormal vulval induction, due to a failure to receive the signal properly. Now, in *Drosophila*, characterization of the role of junctional proteins in DC provides another insight into the regulation of the JNK signaling pathway and may provide new ways to assay for potential ligands and receptors that to date have been elusive in this system.

## Remaining questions

As new molecules are shown to interface with the components of the Rac-JNK signaling cascade, certain

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gaps in our understanding are filled but many more arise with the increasing complexity of interactions. Several compelling problems surrounding the current paradigm for regulation of DC need to be addressed in future studies.

First, the nature of the stimulus for JNK activation during DC is unknown. Direct experimental evidence suggests that active signaling through the JNK pathway is required throughout the progression of DC to maintain the movements, but the observation that LE cells are able to stretch to some degree, although not to the full extent, calls into question the requirement of active JNK signaling for initiation of DC. It is intriguing to speculate that tension, or adhesion, sustained by cell junctions, or contractility itself, mediated by the cytoskeleton, might stimulate the JNK pathway, which then maintains the progression of cellular elongation and movement through regulated feedback mechanisms such as Puc. This so-called mechanotransduction, or coupling of cellular deformation and stretching to the signal transduction machinery, has been documented in mammalian muscle and bone cells and often leads to stimulation of stress signaling cascades (Hamada *et al.*, 1998; Komuro *et al.*, 1996; MacKenna *et al.*, 1998). The source of the proposed signal may also be from tissue neighboring the LE, such as the amnioserosa, but to date, little evidence has accumulated addressing the role of the amnioserosa in DC. Perhaps contraction of the amnioserosa itself triggers LE elongation and DC initiation without the use of a secreted ligand. This may prove to be an area of active research for the future.

Second, and related to the previous issue, there is a disparity in the temporal relationship between JNK pathway activation, using *dpp* as a readout, and the movements of DC. Clearly JNK signaling is crucial for the movements of DC because mutations in JNK pathway components lead to a severe dorsal open cuticle. But in elaborating the role of the JNK pathway, studies have focused on events that occur post-germband retraction, because this is when cellular elongation and morphogenetic movements take place. The events that occur earlier during germband extension and retraction may hold a key to some of the unanswered questions, particularly about the specification of the cell types (LE) and the origin of 'signals' that activate the JNK pathway. The *dpp* expression pattern is refined during stages 11/12 from an earlier broader pattern to a LE-restricted pattern, at about the same time as the germband retracts. This fact emphasizes that the JNK pathway must be active several hours before cellular elongation in the dorsal epidermis commences. It will be important to consider what is happening prior to and during this period of *dpp* pattern refinement and also what takes place just after germ band retraction and the onset of DC. Continuing investigation is necessary to understand how the temporal and spatial confinement of signaling elements is regulated.

Third, how do we make sense of the role of the various Rho family GTPases in DC and in the activation of the JNK pathway? Characterization of Pkn and Mbc begin to raise the possibility that there may be a functional branch in the pathway from Rac to the cytoskeleton independent of the JNK pathway and transcriptional control. The key evidence for this notion is that transcriptional targets of the JNK

pathway are minimally affected in *pkn* and *mbc* mutant embryos but LE cell elongation is blocked and DC still fails (Lu and Settleman, 1999; Nolan *et al.*, 1998). In contrast, multiple lines of evidence suggest that the essential function of Rac is the ultimate activation of Jun/Fos AP-1 and transcriptional regulation mediated through the JNK pathway. For example, mutations in *hep* can suppress activated Rac, and Puc phosphatase overexpression mimics the effects on the LE cytoskeleton of both JNK loss-of-function and expression of DN-Rac (Glise and Noselli, 1997; Martin-Blanco *et al.*, 1998). These data call into question the role of each GTPase in DC and the extent of branching signals upstream of the JNK pathway.

### JNK signaling in tissue polarity

Although DC has provided an excellent system for teasing apart the regulation JNK signaling, additional requirements for *bsk* have been noted in postembryonic developmental processes, suggesting reuse of JNK signaling components in distinct developmental scenarios. For instance, Bsk and several other molecules described in this review contribute to proper development of the adult eye. Comprehensive genetic studies of eye development have elucidated the essential role of the Ras-Raf-MAPK pathway, linked to two receptor tyrosine kinases, sevenless and DER (*Drosophila* EGF receptor), in regulating photoreceptor specification and differentiation (Hafen *et al.*, 1993; Wassarman *et al.*, 1995). Although the role of the JNK pathway in photoreceptor development has been questioned because of lack of phenotypes of *bsk* or *DJun* mutant clones in the eye (Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar *et al.*, 1996), this interpretation may be confounded by redundancy with the MAPK pathway. Alternatively, JNK signaling may not be required for photoreceptor differentiation *per se*, but for a distinct developmental event such as rotation of the eye facets. Indeed, genetic epistasis has placed *bsk*, *hep*, *rhoA* and *dishevelled* (*dsh*) in a pathway downstream of the *Frizzled* (*Fz*) serpentine receptor in generating proper tissue polarity based on dominant suppression, by mutations in these genes, of *Fz*- or *dsh*-induced ommatidial rotation defects (Boutros *et al.*, 1998; Strutt *et al.*, 1997). The connection between *dsh* and *bsk* is further supported by experiments in tissue culture cells which revealed that transfected *dsh* is capable of stimulating JNK activity (Boutros *et al.*, 1998). It remains to be determined whether the linkage between *Fz/dsh* and the JNK signaling cassette has any implications for DC, though it seems unlikely since *dsh* mutations lead to segmentation defects during embryogenesis and do not cause dorsal holes. It is still intriguing to speculate that perhaps DC also relies on proper planar polarity of the embryonic dorsal epidermis in order to coordinate the directed cellular movements of the epithelium toward the midline.

### p38 signaling in development

In the last year and a half several groups have reported the identification of *Drosophila* homologs of components in the p38 MAPK signaling cascade. For





example, two laboratories isolated *DMKK3/licorne* (*lic*), by homology-based screening using a degenerate PCR approach (Han *et al.*, 1998b), and also by functional complementation of the *polymyxin B* sensitive (*pbs2*) knockout in yeast (Suzanne *et al.*, 1999). PBS2 is the MKK for HOG1, the yeast p38 homolog which participates in the high osmolarity response (Herskowitz, 1995). Similar approaches were taken to isolate *Drosophila* p38 genes, for which there are two, *D-p38a* and *D-p38b*. *D-p38a* was isolated by degenerate PCR screening of cDNA libraries (Han *et al.*, 1998a,b) and *D-p38b* was isolated by two groups independently, both for its homology to *D-p38a* (Han *et al.*, 1998b) and for its ability to functionally compensate for HOG1 (Adachi-Yamada *et al.*, 1999).

To examine whether *DMKK3/lic* is required for *Drosophila* development, mutations in the gene were sought. Since *DMKK3/lic* is directly adjacent to *MKK7/hep* on the X chromosome, a *P* element insertion in *hep* was used to generate excision deletions of the two loci (Suzanne *et al.*, 1999). Then, by providing *Hep* function from a transgene, defects specific to loss of *DMKK3/lic* could be analysed in the background of a deficiency for both genes. Given that *DMKK3/lic* transcripts are expressed highly in early precellular blastoderm embryos, and indicate a large maternal contribution, germline clonal analysis was performed to address the function of *DMKK3/lic* in early development. This analysis demonstrated a role of *DMKK3/lic* in several processes, including organization of the posterior pole plasm and abdominal segments in embryos, and establishment of both anterior/posterior and dorsal/ventral axes in the developing oocyte (Suzanne *et al.*, 1999). Eggs laid by mothers mutant in their germline for *DMKK3/lic* were short and round, usually unfertilized, and moderately to severely ventralized, exhibiting a shift in the position of the pair of dorsal breathing appendages of the eggshell toward the dorsal side often to the point of fusion into a single appendage. Although the eggshell is secreted by somatic cells surrounding the germline, which would be heterozygous and functionally wildtype for *DMKK3/lic*, the nonautonomy of the eggshell phenotype suggests that *lic* may affect a secreted signal between the germline and soma. Indeed, *gurken* (*grk*), a TGF- $\alpha$  ligand, establishes the dorsal/ventral axis as a localized signal emanating from the oocyte (Gonzales-Reyes *et al.*, 1995). *Grk* signal is transmitted to the follicle cells via the EGF receptor (*DER*) coupled to the MAPK pathway (Gonzales-Reyes *et al.*, 1995). The dorsal/ventral defect observed in *DMKK3/lic* germline clones occurs as a result of a posttranscriptional effect on *grk* mRNA (Suzanne *et al.*, 1999). *grk* mRNA is properly localized but the protein signal is reduced or diffuses away, resulting in a reduction in *DER* signaling and inability to specify the full range of dorsal fates. The nature of the *lic* effect on *Grk* translation, anchoring, or secretion is still unknown however. Examination of the role of p38 signaling during oogenesis has uncovered a striking connection where one MAPK pathway is used in a specific cell type (germline) to provide a ligand for activation of a different MAPK pathway in an adjacent cell type (somatic follicle cells). It will be very interesting to see if this sort of cellular linkage is working in other developmental phenomena.

Additional phenotypes associated with the small, round shape of the eggshell are characteristic of anterior/posterior polarity defects and failure of the supporting germline nurse cells to dump their contents to the oocyte during the latter stages of oogenesis. These multiple defects raise the question whether the p38 kinase, *DMKK3/lic*, may perform several 'independent' functions during oogenesis, to establish both coordinate axes, or may regulate the activity of downstream effectors that contribute to the overall spatial organization of the developing oocyte and egg chamber, which when compromised, leads to defects in overall axial polarity. It is not clear from these analyses if p38 activation regulates transcription in oogenesis, since the oocyte nucleus is thought to be transcriptionally silent. Nurse cell nuclei are highly transcriptionally active, though, and p38 may control transcription factors that participate in regulation of gene expression whose products are subsequently required in the oocyte. It is formally possible that the defects associated with *DMKK3/lic* loss-of-function result from failure to regulate effectors in the oocyte in a manner independent of the transcriptional machinery, but tissue culture and *in vitro* assays have confirmed the ability of *Drosophila* p38 kinases to phosphorylate and activate mammalian ATF2 (Adachi-Yamada *et al.*, 1999; Han *et al.*, 1998b) suggesting that transcription factors are likely to be physiological targets of p38 activity *in vivo* in fly tissues.

Although mutations in either *D-p38* gene have not yet been reported, transgenic analysis of dominant negative versions and antisense *D-p38b* constructs revealed a role for this protein in wing morphogenesis (Adachi-Yamada *et al.*, 1999). Reduction in *D-p38b* activity appears to disrupt *dpp* signaling specifically. Expression of DN-*D-p38b* can give rise to a wing phenotype resembling *dpp* loss-of-function in the wing, can enhance the phenotype of a weak *dpp* mutant wing, and can suppress the defects associated with hyperactivity of a constitutive form of the *Dpp* receptor, *Tkv*. Consistent with these genetic interactions, various epistasis tests place *D-p38b* downstream of *Tkv*. In larvae expressing activated *Tkv*, only a slight increase in the level of phospho-*D-p38b* is detected. But levels of phospho-*D-p38b* are dramatically increased in cultured fly cells or whole adult flies subjected to heatshock, suggesting that even though p38 activation may be regulated during normal development, activation may also be inducible after stress treatment like heat shock (Adachi-Yamada *et al.*, 1999; Han *et al.*, 1998a). Moreover, both *D-p38a* and *D-p38b* phosphorylation and catalytic activity are upregulated in *Drosophila* S2 cells subjected to UV irradiation (Han *et al.*, 1998b). Continuing investigations into the role of p38 and *DMKK3/lic* in oogenesis, embryogenesis, postembryonic development, and homeostasis will surely an exciting field to follow.

### Stress signaling in immunity

The widespread use of stress signaling pathways in the vertebrate immune system is well documented (Ip and Davis, 1998; Kyriakis and Avruch, 1996). Both JNK and p38/ERK signaling pathways are responsive to inflammatory cytokines and result in transcriptional

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regulation of genes required for the inflammatory response. While research on insect immunity intensifies, it is intriguing to look for parallels in the use of stress signaling in the *Drosophila* immune response. To date, ascertaining whether certain components of stress signaling are utilized *in vivo* during an immune response in the fly has been hampered by the fact that many of the genes cause embryonic lethality precluding analysis of mutant larvae or adults. Although several systems for generating mutant clones of tissue in an otherwise wildtype background have been used with success in various tissues, tools for generating and investigating clones of mutant immune cells have lagged. Nonetheless, initial evidence substantiating a role for JNK and p38 pathway components in *Drosophila* immunity has come from insect cell culture systems. Treatment of a *Drosophila* mbn-2 cell line, derived from macrophage-like hemocytes, with bacterial LPS (endotoxin), which is capable of eliciting a physiological response *in vivo*, leads to rapid stimulation of Bsk (JNK) activity monitored by an *in vitro* kinase assay with its substrate DJun (Sluss *et al.*, 1996). Similarly, LPS can stimulate phosphorylation and activity of D-p38a and D-p38b in transfected cells (Han *et al.*, 1998a,b). Thus, both stress pathways appear to be induced by inflammatory stimuli in *Drosophila* cultured cells. Interestingly, an anti-inflammatory drug, SB203580, that has been shown to specifically target and disable human p38 kinases, is capable of inhibiting the catalytic activity of the *Drosophila* p38 isoforms *in vitro* (Han *et al.*, 1998b). Furthermore, addition of this drug to LPS-treated p38-transfected S2 cells significantly prolongs the LPS response, measured by antimicrobial peptide target gene expression, suggesting that inhibition of p38 function may potentiate the immune response in the fly, contradicting the presumed role of this drug for anti-inflammation. To test the hypothesis that p38s may play a role in downregulation of the immune response *in vivo*, a transgenic *D-p38a* construct was expressed under an inducible heat shock promoter in larvae that were subsequently subjected to bacterial challenge. *D-p38a* overexpression resulted in suppression of an immune response, again measured by the levels of target gene expression (Han *et al.*, 1998b). Taken together, these data strongly suggest that p38s can be induced by an immune challenge but then serve to downregulate or attenuate the response, so as not to prolong inflammation. Carefully designed mutant screens to identify molecules functioning in the immune system have

recently been conducted and may uncover new (viable) alleles of stress signaling components deficient in immune response (Wu and Anderson, 1998). This sort of genetic analysis will certainly prove valuable to confirm the role of JNK and p38 signal transduction pathways in insect immunity and to shed light on relevant tissue-specific signals and responses.

### Concluding remarks

*Drosophila* is no exception when it comes to the presence and use of multiple MAPK signal transduction cascades during development and homeostasis. We have discussed how signaling cascades in the fly functionally related to the stress induced signaling pathways in vertebrates are utilized repeatedly in different cellular and developmental contexts, with distinct inputs and outcomes. Major roles for the JNK pathway in *Drosophila* include the regulation of complex morphogenetic movements during embryogenesis and generation of tissue polarity in the adult. The recently identified p38 pathway also appears to participate in morphogenetic processes and the generation of polarity in the egg chamber. Preliminary evidence also sets the stage for further characterization of JNK and p38 cascades in insect immunity.

One could imagine that morphogenesis, or the generation of form and shape in the context of multicellular organisms, such as that described in *Drosophila* DC, is an extension of the use of the same conserved pathways operating in unicellular yeast to control membrane and cytoskeletal organization and thus cell shape (osmotic response, mating protrusion), polarity (bud site selection), cell cycle, growth and differentiation. Furthermore, not only are the sequence and order of molecules in signaling cassettes like the JNK and Dpp pathway conserved, but the use of these two pathways sequentially to drive major morphogenetic movements has been documented from fly to man. The diverse processes that utilize MAPK and the stress signaling pathways, in particular, clearly underscore the exquisite versatility and specificity provided by the use of multiprotein phosphorylation cassettes throughout evolution. As in most scientific endeavors, new answers lead to new questions. Investigating the role of stress signaling genetically in *Drosophila* has stimulated a whole field of inquiry into regulation of tissue morphogenesis and polarity during development.

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# Sequential Activation of Signaling Pathways during Innate Immune Responses in *Drosophila*

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## Summary

Innate immunity is essential for metazoans to fight microbial infections. Genome-wide expression profiling was used to analyze the outcome of impairing specific signaling pathways after microbial challenge. We found that these transcriptional patterns can be dissected into distinct groups. We demonstrate that, in addition to signaling through the Toll and Imd pathways, signaling through the JNK and JAK/STAT pathways controls distinct subsets of targets induced by microbial agents. Each pathway shows a specific temporal pattern of activation and targets different functional groups, suggesting that innate immune responses are modular and recruit distinct physiological programs. In particular, our results may imply a close link between the control of tissue repair and antimicrobial processes.

## Introduction

Innate immunity is an ancient biological process that multicellular organisms use for their defense against pathogenic challenges. First-line defense mechanisms are found in various evolutionarily distant metazoans, from plants to humans (reviewed in Hoffmann et al., 1999). In higher vertebrates, innate immunity is an integral part of the host defense system that triggers the expression of costimulatory molecules, whereas invertebrates rely exclusively on innate mechanisms for protection against infectious agents (Schnare et al., 2001; reviewed in Akira et al., 2001; Fearon and Locksley, 1996; Janeway, 1989).

In recent years, Toll-like receptors (TLRs) have been shown to play a crucial role in innate immunity (reviewed in Aderem and Ulevitch, 2000; Kimbrell and Beutler, 2001). Toll was initially identified in *Drosophila* to be required for the establishment of embryonic dorsoventral polarity (Anderson et al., 1985). Later studies demonstrated that Toll mediates fungal and gram-positive-specific immune responses (Lemaitre et al., 1996). In mammals, TLRs recognize pathogen-specific markers, such as lipopolysaccharides (TLR4), peptidoglycans (TLR2), and viral double-stranded RNA (TLR3; Alexopoulou et al., 2001; Hayashi et al., 2001; Poltorak et al., 1998; Takeuchi et al., 1999). In *Drosophila*, two distinct NF $\kappa$ B

signaling pathways are activated upon microbial infection. In response to gram-positive and fungal infection, Toll triggers a transcriptional response through the death domain-containing protein Tube (Tub), the IRAK-like protein kinase Pelle (Ple), and the I $\kappa$ B-like inhibitor factor Cactus (Cact; Lemaitre et al., 1995b, 1996). The inactivation of Cact leads to the translocation of the NF $\kappa$ B transcription factor Dif and induction of antimicrobial target genes. Gram-negative bacteria signal through a distinct pathway, referred to as the Imd pathway (Lemaitre et al., 1995a). Downstream of a receptor, the signal is relayed by Imd, IKK $\beta$ , and IKK $\gamma$  (encoded by *key*) and Relish (Rel) (Georgel et al., 2001; Hedengren et al., 1999; Lu et al., 2001; Silverman et al., 2000; Vidal et al., 2001).

One of the fundamental questions in signal transduction is, how are extracellular signals transmitted through a network of components to control specific cellular functions? Many signaling pathways, such as Toll pathways, are reutilized for different cellular programs. However, the mechanisms by which pathways establish their specificity remains largely unresolved. Microarray analysis has been widely used to investigate changes in gene expression levels that may be indicative of specific biological programs. In yeast, expression analysis has revealed that genes operating in a common process, such as mitosis or replication, are often tightly coregulated (Chu et al., 1998). Recently, several studies have investigated the transcriptional response to microbial infections in *Drosophila* (De Gregorio et al., 2001; Irving et al., 2001). While it is well established that NF $\kappa$ B-dependent signaling pathways play a central role in innate immune responses, the role of other signaling events remains poorly understood.

Here, we use expression profiling and loss-of-function experiments to analyze signaling pathways that control microbial challenge-induced transcriptional patterns. We find that dynamic patterns after LPS stimulation and septic injury can be dissected into distinct, signaling pathway-specific responses. These signaling signatures appear with discrete temporal patterns, revealing a link between pathway activation and temporal organization that can be used to predict the activation of distinct signaling pathways. Our data indicates that, in addition to the Toll and Imd pathways, the JNK and JAK/STAT pathways contribute to the expression of microbial challenge-induced genes. Each pathway's target set is enriched for genes of specific functional groups, indicating that these signaling pathways fulfill separate functions after microbial infection. Our results reveal a connection of pathway targets and temporal patterns and indicate a close link between antimicrobial peptide expression and the induction of cytoskeletal remodeling.

## Results

### Signaling in Response to Lipopolysaccharides

Lipopolysaccharides (LPS) are the principal cell wall components of gram-negative bacteria. In mammals,

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exposure to LPS causes septic shock through a TLR4-dependent signaling pathway (Poltorak et al., 1998). LPS treatment of *Drosophila* SL2 cells leads to rapid expression of antimicrobial peptides, such as Cecropins (Cec; Samakovlis et al., 1992). SL2 cells resemble embryonic hemocytes and have also been used as a model system to study JNK and other signaling pathways (Han and Ip, 1999; Sluss et al., 1996). LPS-responsive induction of the antimicrobial peptides AttacinA (AttA), Diptericin (Dipt), and Cec was shown to rely on IKK and Relish (Silverman et al., 2000). In order to obtain a broad overview on the transcriptional response to LPS in *Drosophila*, we generated genome-wide expression profiles of SL2 cells at different time points following LPS treatment.

Figure 1A shows the hierarchical clustering of 238 genes that passed our filtering criteria (see Experimental Procedures). In time-course experiments, we observed a complex pattern of gene expression that can be separated into different temporal clusters. A first group, with peak expression at 60 min after LPS, primarily consists of cytoskeletal regulators, signaling, and proapoptotic factors. This group includes cytoskeletal and cell adhesion modulators such as Matrix metalloprotease-1, WASp, Myosin, and Ninjurin, proapoptotic factors such as Reaper, and signaling proteins such as Puckered and VEGF-2. A second group, with peak expression at 120 min, includes many known defense and immunity genes, such as Cec, Mtk, and AttA, but not the gram-positive-induced peptide Drs. Interestingly, this cluster also includes PGRP-SA, which is a gram-positive pattern recognition receptor *in vivo* (Michel et al., 2001), suggesting possible crossregulation between gram-positive- and gram-negative-induced factors. A third group is transiently downregulated upon LPS stimulation. This cluster includes genes that play a role in cell cycle control, such as String and Rca1 (see also Figure 1B and Supplemental Data at <http://www.developmentalcell.com/cgi/content/full/3/5/711/DC1>). Altogether, our results show that, in response to LPS, a defined gram-negative stimulus, cells elicit a complex transcriptional response.

#### JNK and Rel Signals Branch Downstream of Tak1

In adult *Drosophila*, gram-negative bacteria elicit an antimicrobial response mediated by a signaling pathway that involves the intracellular factors Imd, Tak1, Key, and Rel (Georgel et al., 2001; Hedengren et al., 1999; Lu et al., 2001; Vidal et al., 2001). On the basis of our previous results, we reasoned that the temporal waves of transcriptional activity in SL2 cells might reflect different signaling pathway contributions. We therefore asked whether selectively removing signaling components by RNA interference (RNAi) would block induction of all, or only parts, of the transcriptional response to LPS.

We first investigated the effect of removing *key* or *rel* by RNAi. As shown in Figure 2A, the expression profiles demonstrated that removing *key* or *rel* diminished the induction of antimicrobial peptides. However, the induction of cytoskeletal and proapoptotic factors was not affected. In contrast, removing *tak1* reduced the level of induction or repression for all identified genes (Figure 2A), indicating that LPS-induced signaling is transmitted through Tak1 and that specific pathways branch downstream of Tak1.

In the Rel-independent group, we identified several transcripts that were indicative of other signaling events. For example, *puc* is transcriptionally regulated by JNK signaling during embryonic development (Martin-Blanco et al., 1998). Therefore, we tested the effect of removing SAPK/JNK activity on LPS-induced transcripts. As shown in Figure 2A, *mkk4/hep* dsRNA-treated cells lose the ability to induce the Rel-independent cluster, indicating that LPS signaling branches downstream of Tak1 into separate Rel- and JNK-dependent branches (see also Figure 2B). To validate the results obtained from the microarray experiments, we performed quantitative PCR (qPCR) using *puc* and *cec* mRNA levels as indicators for Imd/Rel- or Mkk4/Hep-dependent pathways. Additionally, we tested the effect of removing *imd*, which, *in vivo*, acts upstream of Tak1, to clarify whether, in addition to Tak1, other known upstream components of a gram-negative signaling pathway are required for both Rel- and Mkk4/Hep-dependent pathways (Georgel et al., 2001; Vidal et al., 2001). These qPCR experiments confirmed that *cec* is dependent for its expression on Imd, Tak1, Rel, and Key, whereas LPS-induced *puc* expression is dependent on Imd, Tak1, and Mkk4/Hep (Figure 2C). Hence, the immunity signaling pathway in response to LPS bifurcates downstream of Imd and Tak1 into Rel- and SAPK/JNK-dependent branches. Both the Rel and SAPK/JNK pathways regulate different functional groups of downstream target genes.

While both Rel and Mkk4/Hep pathways are downstream of Imd and Tak1 in response to LPS, the two downstream branches elicit different temporal expression patterns. We then asked whether the first transcriptional response is controlled by downstream targets that might negatively feed back into the signaling circuit. *puc* was a candidate for such a transcriptionally induced negative regulator. We therefore compared expression profiles of cells depleted for *puc* before and after a 60 min LPS treatment. As shown in Figure 3A, these experiments showed that transcripts dependent on the Mkk4/Hep branch of LPS signaling were upregulated, even without further LPS stimulus. In contrast, Rel branch targets were not influenced (Figure 3A). We also noticed that *puc* dsRNA-treated cells show loss of the typical round cell shape. These cells appear flat and have a delocalized Actin staining (Figure 3B), consistent with a deregulation of cytoskeletal modulators in *puc*-deficient cells.

The analysis of expression profiles showed that, while SAPK/JNK and Rel signaling are controlled by the same Imd/Tak1 cascade, they appear to have different feedback loops. Whereas Rel signaling induces Rel expression and thereby generates a self-sustaining loop, possibly leading to the maintenance of target gene expression, the SAPK/JNK branch induces an inhibitor and thereby establishes a self-correcting feedback loop. These results may explain how a single upstream cascade can lead to different dynamic patterns.

#### Septic Injury Induces Multiple Distinct Temporal Sets of Target Genes

Septic injury of adult *Drosophila* is a widely used model system to study innate immune responses *in vivo* (Le-maitre et al., 1995a, 1996). To explore the signaling



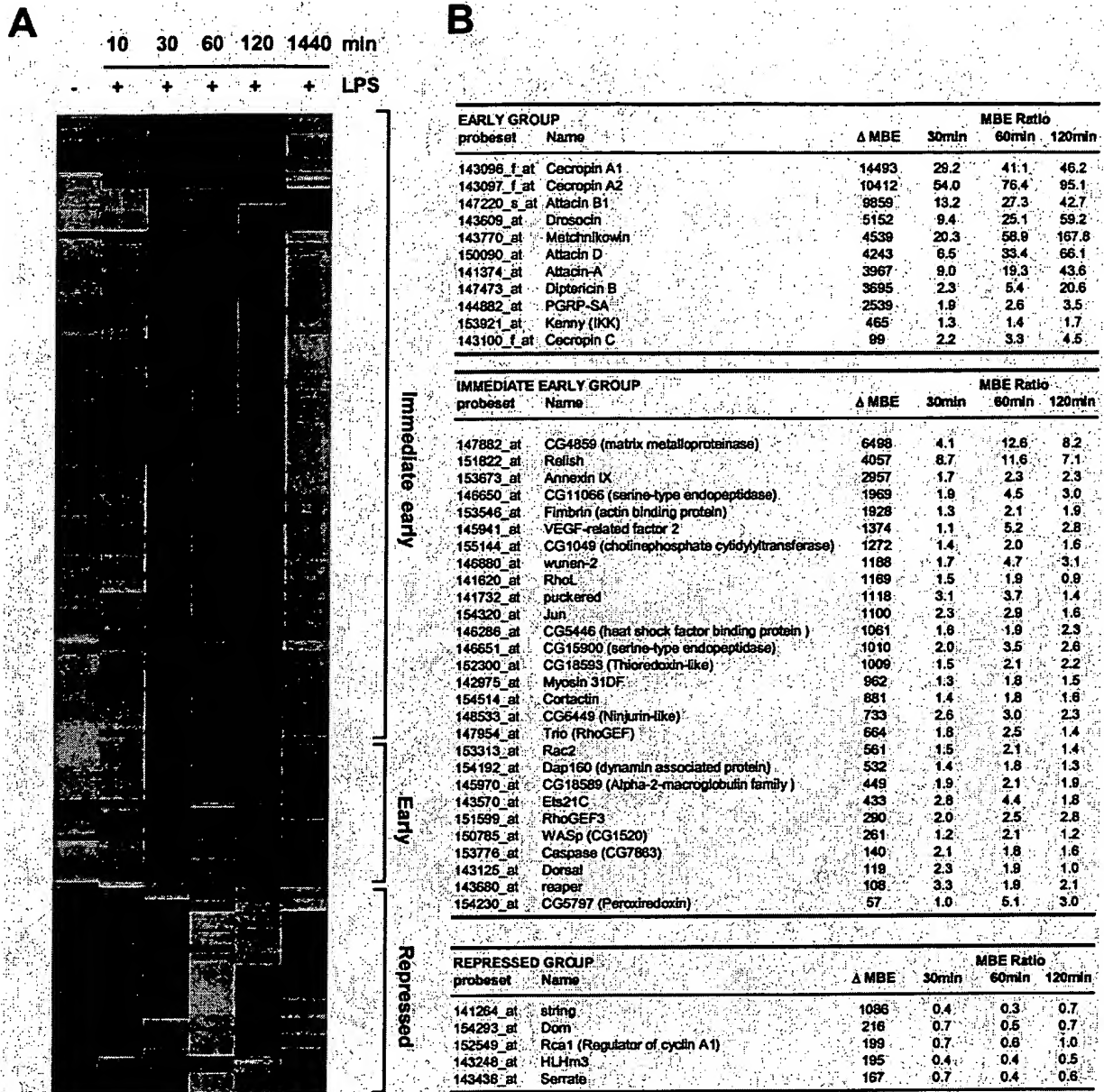


Figure 1. Lipopolysaccharides Induced Expression Patterns in SL2 Cells

(A) Hierarchical clustering diagram of 238 filtered genes. Expression profiles were generated after LPS treatment for the indicated times. Data analysis was performed with a model-based algorithm implemented in dChip. Expression values in gene rows are mean centered, normalized to have a variation of 1, and hierarchically clustered (Eisen et al. 1998). Different shades of green represent model-based expression values between 0 and 3 standard deviations below the mean value, and shades of red represent expression values between 0 and 3 standard deviations above the mean value. Note that "immediate early" and "early" clusters are clearly distinguished by the timing of their peak expression levels.

(B) Table of differentially regulated genes.  $\Delta$ MBE indicates the difference between the highest and lowest expression values. The column "MBE Ratio" shows the ratio of expression at the indicated time point compared with nonstimulated cells. Note that genes in the early cluster show peak expression at 120 min after LPS treatment, whereas genes in the immediate early cluster show peak expression at 60 min or earlier. See Supplemental Data at <http://www.developmentalcell.com/cgi/content/full/3/5/711/DC1> for a complete listing.

pathways that control induced genes in vivo, we first generated genome-wide expression profiles of adult *Drosophila* infected by septic injury. Equal numbers of male and female adult Oregon R flies were infected with a mixture of *E. coli* (gram negative) and *M. luteus* (gram positive). Subsequently, flies were collected at 1, 3, 6, 24, 48, and 72 hr time points post-septic injury to measure

temporal changes in gene expression levels. Computational analysis identified a list of 223 genes that were differentially regulated and matched our filtering criteria for at least two time points after microbial infection (see Experimental Procedures). This set includes 197 genes that are transiently upregulated and 26 that are transiently downregulated upon immune challenge (see

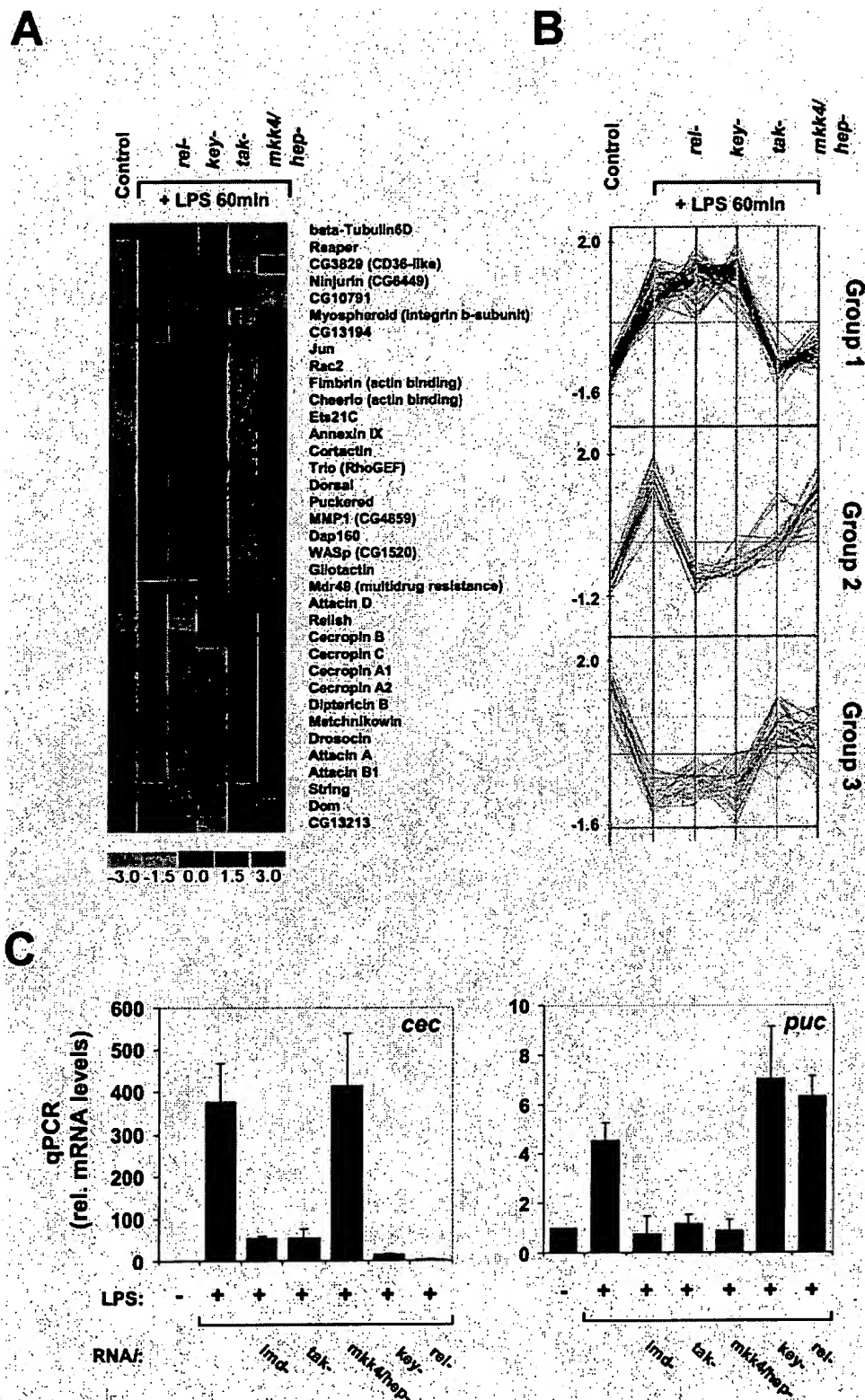
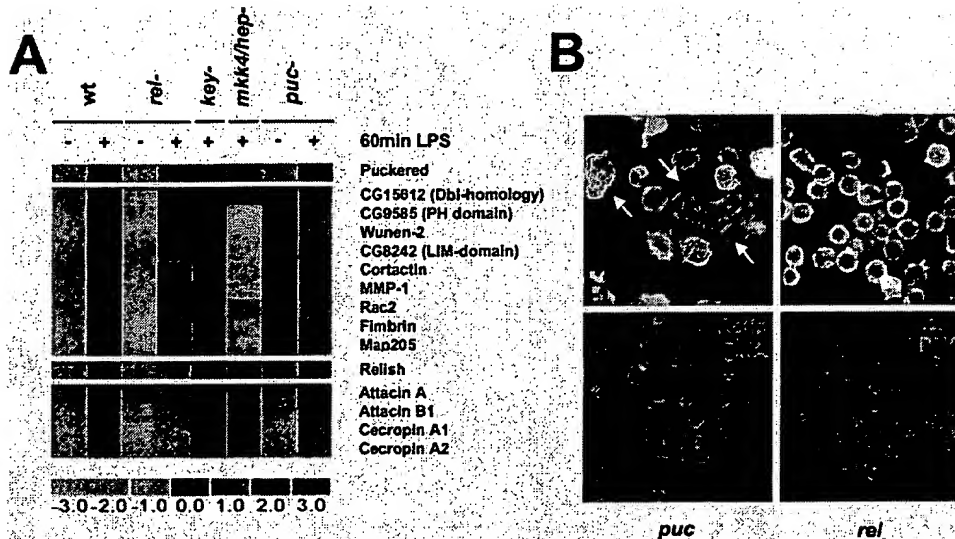


Figure 2. Differential Requirement of Cytoskeletal and Antimicrobial Genes for SAPK/JNK and Rel Pathways

(A) Representation of relative expression levels of Key/Rel- and Mkk4/Hep-dependent transcripts. Cells were treated with the indicated dsRNA for 72 hr prior to a 60 min LPS stimulation. Extracted total RNA was labeled and hybridized to oligonucleotide arrays. Depletion of *tak1* reduces the expression of all LPS-inducible transcripts, whereas depletion of *rel* or *mkk4/hep* abolishes the expression of separate sets of induced transcripts. Color scales are as described in Figure 1.

(B) Overall expression patterns of three differentially regulated groups of genes. Cells were treated for 72 hr with dsRNA against *tak1*, *key*, *rel*, and *mkk4/hep*. Cells were then stimulated for 60 min with LPS. Ratios of expression values were normalized over their variance. Genes





**Figure 3. Puckered Negatively Controls the SAPK/JNK Branch of LPS Signaling and Derepresses Cytoskeletal Genes**

(A) Representation of model-based expression levels. Colors are as described in Figure 1. Removal of *puc* in SL2 cells leads to the constitutive expression of SAPK/JNK pathway-dependent genes, even without LPS stimulus. In contrast, removing *rel* without LPS stimulation does not lead to constitutive expression of cytoskeletal genes.

(B) *puc* removal induces cell shape changes. SL2 cells were treated with dsRNA against *puc* or *rel*. Cells were subsequently washed in PBS, fixed in 4% formaldehyde for 20 min at room temperature, and permeabilized with PBST. Actin was visualized with Alexa 568 phalloidin (Molecular Probes), and DNA was visualized by DAPI (Sigma) staining.

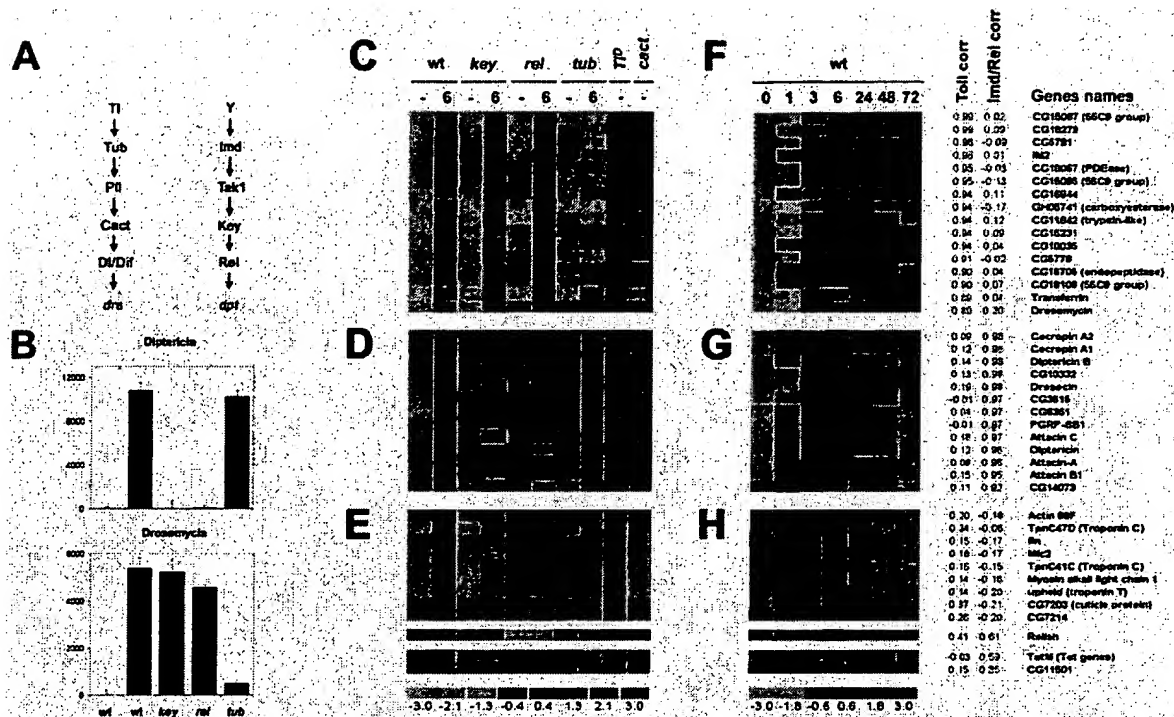
Supplemental Figure S3). Different temporal profiles of gene expression can be detected in our analysis; clusters of genes differed significantly in the timing and persistence of induction. For example, whereas many genes are expressed transiently shortly after infection (see Supplemental Figure S3), others are induced late and are still upregulated at a 72 hr time point. A significant number of genes of both early and late clusters were differentially expressed at a 6 hr time point after infection, which we chose for further analysis.

We then examined the signaling requirements for these differentially expressed transcripts in mutant alleles of known Toll and Imd/Rel pathway components, reasoning that we might uncover additional pathways by analyzing patterns that cannot be reconciled with expected signaling patterns. Flies homozygous for loss-of-function mutations in *tub*, *key*, or *rel* were infected with gram-negative and gram-positive bacteria, and expression profiles were generated for a 6 hr time point after infection. In addition, noninfected *T1<sup>10b</sup>*, a gain-of-function allele of the receptor, and *cact*, a homolog of the inhibitory factor IκB, were used to monitor transcripts that are constitutively expressed in gain-of-function signaling mutants. The antimicrobial peptides *dipt* and *drosomycin* (*drs*) are representative targets for the Toll and Imd/Rel pathways, respectively (Lemaitre et al.,

1995a, 1996). As previously shown, *dipt* induction is not detectable in our expression profiles in either a *rel* or *key* mutant background, whereas its expression is not affected in *tub* mutants (Figure 4B). In contrast, *drs* relies on Tub to convey a Toll-dependent signal. Consistently, our expression profiles show that, in a *tub* mutant background, *drs* expression is diminished (Figure 4B). These experiments showed that the analysis of our mutant expression profiles can be used to deduce signaling requirements for distinct target groups.

Toward a computational annotation of signaling pathways, we employed a pattern-matching strategy to rank transcripts by similarity to bona fide Toll or Imd/Rel pathway targets, such as *dipt* and *drs*. We analyzed a set of 91 transcripts that matched our filtering criteria for differential expression at a 6 hr time point after septic injury. To determine their dependence on known immunity signaling pathways, we calculated the correlation coefficients of the individual gene expression level in mutant backgrounds to binary Toll or Imd/Rel patterns (Table 1). Genes were subsequently ordered according to their correlation coefficients for each pathway signature. Using this strategy, we separated transcripts that primarily belong to either the Toll or Imd/Rel pathway groups (Figures 4C and 4D). For example, genes that show a high correlation coefficient for a Toll pathway

in group 1 are independent of Rel and Key but are dependent on Tak and MKK4/Hep for their induction after LPS treatment. Group 2 genes are expressed independently of MKK4/Hep but are dependent on Tak, Rel, and Key for their expression. Group 3 genes are repressed after LPS treatment. The repression is not affected by Rel and Key, but they are not repressed by Tak and are partially repressed by MKK4/Hep. (C) Quantitative PCR analysis for *cec* (left panel) and *puc* (right panel) as indicators for Rel and SAPK/JNK signaling pathways. Cells were treated with dsRNA for *imd*, *tak1*, *key*, *rel*, and *mkk4/hep* and harvested for total RNA extraction. Left panels show fold changes (FC) calculated from quantitative PCR results with *rp49* as internal normalization control. Treatment with both *mkk4* and *hep* dsRNA was necessary to reduce *puc* expression. Depletion of either *mkk4* or *hep* alone led to a partial reduction of induced *puc* levels (data not shown).



**Figure 4. Signaling Pathway Requirements Correlate with Distinct Temporal Expression Profiles**

**(A) Schematic of Toll and Imd/Rel pathways activated during innate immunity in *Drosophila*.**

(B) Expression values of *dipt* and *drs* in different genetic backgrounds. Differences between expression levels in infected and noninfected *Drosophila* wild-type or mutants were plotted. wt, Oregon R; *key*, *key*<sup>1</sup>/*key*<sup>1</sup>; *rel*, *rel*<sup>E20</sup>/*rel*<sup>E20</sup>; *tub*, *tub*<sup>118</sup>/*tub*<sup>228</sup>; *cact*, *cact*<sup>E9</sup>/*cact*<sup>A2</sup>; *Tp*, *Tp*<sup>106</sup>/+.

(C-E) A representation of model-based expression levels. The left panels show the expression levels of genes in different mutant backgrounds. The right panels show the expression levels during the time course of infection. Expression values in gene rows in each panel were mean centered and variance normalized. Different shades of green represent an expression value between 0 and 3 standard deviations below the mean expression value, and shades of red represent an expression value between 0 and 3 standard deviations above the mean expression value. The column "Toll-corr" shows the correlation coefficient of the indicated genes with the binary Toll signaling pattern in Table 1. The column "lmd/Rel-corr" shows the correlation coefficient of the indicated genes with a bona fide lmd/Rel target gene. A value of 1 indicates a perfect match. A cutoff of 0.75 was used for selecting genes in (C) and (D) (see Supplemental Figure S4 at <http://www.developmentalcell.com/cgi/content/full/3/5/711/DC1> for full diagrams). Both panels show the highest-scoring genes ranked according to their correlation coefficients.

(C) Genes that score high for similarity with the Toll pathway, but low for the lmd/Rel pathway signature. These transcripts are dependent on *tub* for their induction after microbial challenge and are constitutively expressed in *Tp*<sup>106</sup> and *cac*, but they are not affected in *key* and *rel* mutants.

(D) Genes rank high for Imd/Rel, but low for Toll signature similarity. These transcripts are dependent on *key* and *rel* for their induction after microbial challenge, are independent of *tub*, and are not constitutively expressed in *Tr<sup>100</sup>* and *cac*.

(E) Transcripts that score low for both Toll and Imd/Rel pathway signatures.

(F–H) Corresponding temporal patterns.

pattern include *drs*, *transferrin*, a secreted iron binding protein, IM2, and a cluster of homologous secreted peptides at 55C9 (Figure 4C). These genes have a low correlation coefficient for an Imd/Rel pattern, indicating that they are primarily dependent on Toll pathway signaling in response to microbial infection. In contrast, the genes shown in Figure 4D score low for a Toll pathway pattern but have high correlation coefficients for an Imd/Rel pattern. This group includes known gram-negative antimicrobial peptides, such as *cec* and *dipt*, *peptidoglycan receptor-like* genes (*PGRP-SD*, *PGRP-SB1*), other small transcripts (*CG10332*), and genes coding for putative transmembrane proteins, such as *CG3615* (see Supplemental Data for complete listing).

Interestingly, some genes do not fit either pattern, suggesting that they are regulated by other pathways. One group of genes, including cytoskeletal factors such

as *actin88F*, *flightin*, and *tpnC41C*, is induced in  $Tl^{100}$ , but not in *cact* mutants. In contrast, *totM* (Ekengren and Hultmark, 2001) and *CG11501* are expressed at high levels in *cact* mutant flies but are not expressed in  $Tl^{100}$  mutant flies. In addition, these transcripts are highly inducible in a *tub* genetic background, but they are not inducible in *key* or *rel*. This may suggest that Toll, Tub, and Cact do not act in a linear pathway under all circumstances. Moreover, *rel* shows an expression pattern suggesting that it is regulated by both the Imd/Rel and Toll pathways (Figure 4E). Thus, these results indicate that, in addition to the canonical Toll and Imd pathways, other signaling events and possibly signaling pathway branching contribute to the complex expression patterns after septic injury (see derived patterns in Table 1). Finally, we note that there is a strong correlation between pathway requirement and temporal expression

Table 1. Binary Patterns for Assigning Targets to Known and Derived Signaling Pathway Signatures

Pathway Patterns	wt	wt (6 hr)	key	key (6 hr)	rel	rel (6 hr)	tub	tub (6 hr)	Tl10b	cact	Number of Genes	Examples
<b>Search patterns</b>												
Toll group	0	1	0	1	0	1	0	0	1	1	28	<i>drs, im2, CG16836</i>
imd/rel group	0	1	0	0	0	0	0	1	0	0	22	<i>cec, dpt, attA, CG3615</i>
<b>Derived patterns</b>												
cyto group	0	1	0	0	1	1	0	0	1	0	12	<i>act88F, fln, mlc, CG7214</i>
totM group	0	1	0	0	0	0	0	1	0	1	2	<i>totM, CG11501</i>
<b>Others</b>												
Repressed											4	<i>Sodh-1, CG18030</i>
Other											23	<i>relish, CG18589</i>

For each pathway signature, binary expression levels in different mutant backgrounds are listed. Search patterns were used to calculate correlation coefficients and rank transcripts in descending order. We set a cutoff of 0.75 for genes to be counted in the "Number of Genes" column. New patterns from transcripts that could not be assigned to either Toll or Imd/Rel target patterns. Genes that did not match the criteria and have not been assigned are listed in "Others." For a complete listing and expression levels, see Supplemental Data at <http://www.developmentalcell.com/cgi/content/full/3/5/711/DC1>.

pattern. Whereas Toll targets are exclusively found in the sustained cluster, Imd/Rel targets are expressed early and transiently after septic injury. The two additional clusters with noncanonical patterns show temporal patterns distinct from either Toll or Imd pathways (Figures 4F–4H).

#### JNK and JAK/STAT Signaling Control Subsets of Septic Injury-Induced Genes

We reasoned that the patterns observed in our mutant analysis might reflect the contributions of additional signaling pathways. Also, these noncanonical clusters

show distinct temporal expression patterns (Figure 4E and Table 1), suggesting that they are separately controlled. One group of genes consists primarily of cytoskeletal regulators and structural proteins that are expressed early on, with peak expression at 3 hr. These include several muscle-specific proteins, thus possibly reflecting the organ that is injured during injection. For example, *fln* encodes a cytoskeletal structural protein expressed in the indirect flight muscle (Reedy et al., 2000).

Since our previous results demonstrated that the expression of cytoskeletal genes after LPS stimulation is

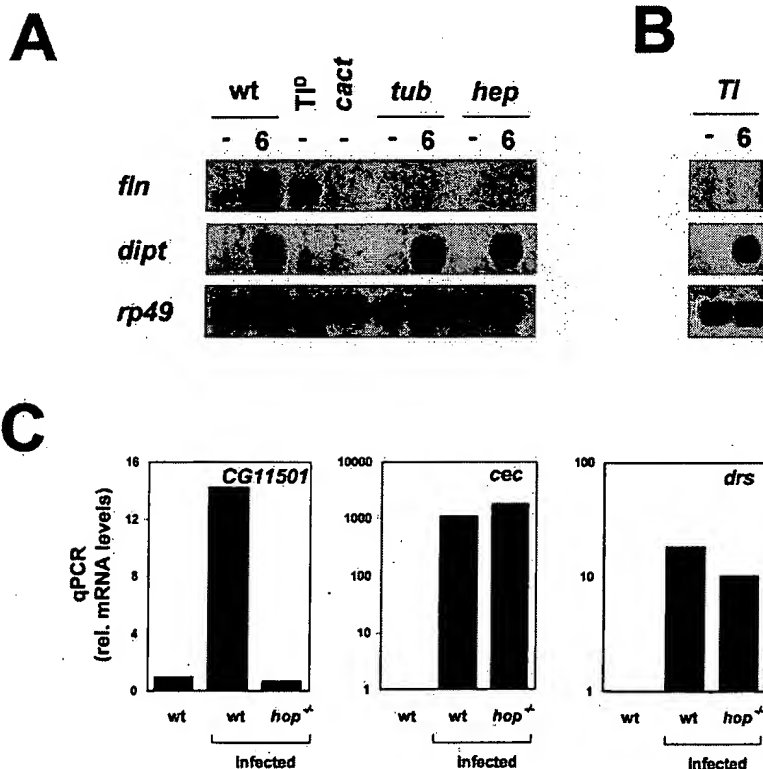


Figure 5. JNK and JAK/STAT Signaling Control Subsets of Genes during Septic Injury Responses

(A–B) Northern Blot analysis of mutants in Toll and JNK pathways (20 µg of total RNA per lane). The same blot was probed with a *fln*-specific probe, stripped, reprobed with a *dpt* probe, and subsequently reprobed with an *rp49* probe as a loading control. Genotypes used were as follows: wt, Oregon R; *tub*, *tub*<sup>118/tub</sup><sup>228</sup>; *cact*, *cact*<sup>69/cact</sup><sup>42</sup>; *Tl*<sup>0</sup>, *Tl*<sup>10b/+</sup>; *Tl*, *Tl*<sup>44/Tl</sup><sup>PORE</sup>; *hep*, *hep*<sup>1</sup>.

(C) Quantitative PCR analysis for CG11501, *cec*, and *drs* in wt and *hop* (*hop*<sup>navi</sup>/*hop*<sup>438</sup>) genetic backgrounds.

dependent on a JNK cascade, we examined whether removing JNK activity *in vivo* affects the induction of *fln*. In *Drosophila*, JNK signaling pathways have been previously implicated in epithelial sheet movements during embryonic and pupal development, a process that has been likened to wound-healing responses (Jacinto et al., 2001; Ramet et al., 2002). Our results indicate that in *hep*<sup>1</sup> (JNKK) mutants, which are impaired in JNK signaling (Glise et al., 1995), the induction of *fln* is diminished, whereas the expression of the antimicrobial peptide *dipt* is not affected (Figure 5A). We then tested whether *fln* induction in *Tl* loss-of-function alleles is affected. These experiments showed that *fln* expression is lost in *Tl* mutants, suggesting that Toll acts upstream of a JNK pathway to induce septic injury-induced target genes (Figure 5B).

The clustering revealed a second noncanonical group with small proteins that are expressed late and transiently with peak expression at 6 hr after septic injury. One of the clustered transcripts, CG11501, encodes a small Cys-rich protein that is 115 amino acids long and is strongly induced after septic injury. By RT-PCR, we confirmed that CG11501 is upregulated after septic injury (Figure 5C). In order to characterize how CG11501 is controlled after microbial challenge, we first undertook a candidate pathway approach. In an independent study, we found that *totM* gene induction, which is part of the same cluster, is dependent on a JAK/STAT signaling pathway (Agaisse et al., in preparation). We therefore examined whether CG11501 induction requires JAK/STAT signaling. Mutations in JAK/STAT pathways in *Drosophila* have been implicated in various processes during embryonic and larval development (Perrimon and Mahowald, 1986; Hou and Perrimon, 1997). In *Anopheles*, STAT is activated in response to bacterial infection (Barillas-Mury et al., 1999). Similarly, gain-of-function STAT has been implicated in the transcriptional control of thiolester proteins (Lagueux et al., 2000). We examined here mutant alleles of *hopscotch* (*hop*), the *Drosophila* homolog of JAK. Quantitative PCR shows that CG11501 induction after septic injury is diminished in *hop* loss-of-function mutants, whereas the expression of Toll and Imd targets *drs*, and *cec* is not affected (Figure 5C).

## Discussion

In this study, we have investigated the signaling circuits during innate immunity in adult *Drosophila* and cells. Our results show that, in addition to NF $\kappa$ B signaling pathways, other pathways direct the expression of functionally distinct groups of target genes. Specifically, we show that, in addition to known innate immune cascades, JNK and JAK/STAT are required for the transcriptional response during microbial challenge. We find that one transcriptional signature of small secreted peptides can be traced to JAK/STAT signaling. Additionally, JNK signaling controls cytoskeletal genes after an LPS stimulus and after septic injury *in vivo*. Both in cells and *in vivo*, JNK pathways are connected to the same upstream signaling cassette that induces NF $\kappa$ B targets. Altogether, these results suggest that innate immune signaling pathways closely link cytoskeletal remodeling,

as required for tissue repair, and direct antimicrobial actions. Our data also provide insights into the connection of temporal patterns and the activation of distinct signaling pathways.

## NF $\kappa$ B-Independent Signaling during *Drosophila* Innate Immunity

NF $\kappa$ B pathways play a central role for innate and adaptive immune response in mammals. In innate immune responses, TLRs on dendritic cells recognize microbial agents and activate NF $\kappa$ B, leading to the expression of proinflammatory cytokines and other costimulatory factors required to initiate an adaptive immune response. Additionally, other signaling pathways have been implicated at later stages during immune responses in mammals, but their physiological role in innate immunity remains rather poorly understood. For example, several cytokines, such as IL-6 and IL-11, signal through a JAK/STAT pathway to induce the expression of acute phase proteins (reviewed in Ihle, 2001). Similarly, JNK pathways are activated in response to TNF and IL-1, may lead to the expression of immune modulators, and are required for T cell differentiation (Hambleton et al., 1996; Dong et al., 2000). In *Drosophila*, studies have investigated two distinct NF $\kappa$ B-pathways—Toll and Imd/Rel—that have been shown to mediate gram-positive/fungal and gram-negative responses. Both pathways induce specific antimicrobial peptides and thereby focus the response on the invading microbial agent. Genetic analysis has shown that functions of the NF $\kappa$ B-pathways are separable; *Drosophila* that are mutant for only one of these pathways are susceptible to subgroups of pathogens (Lemaître et al., 1995a). We have asked here whether we can identify the contribution of NF $\kappa$ B-dependent and, possibly, other signaling pathways by examining global expression profiles. The obtained data set demonstrated that NF $\kappa$ B-independent signaling pathways contribute to the transcriptional patterns observed after microbial infection. Both in cells and *in vivo*, JNK-dependent targets precede the peak expression of antimicrobial peptides that require NF $\kappa$ B. JAK/STAT targets are induced with a distinct temporal pattern that shows late, but only transient, expression characteristics (Figure 6). The stereotyped pathway patterns after microbial challenge suggest that the correct temporal execution of signaling events, similar to signaling during development, may play an important role in the regulation of homeostasis.

## JNK Signaling Controls Cytoskeletal Gene Expression

Strikingly, our experiments show that cytoskeletal gene expression during innate immune responses is controlled by JNK through the same upstream signaling cascade that activates NF $\kappa$ B pathways. JNK pathways have been implicated in a variety of biological responses. In mammals, cell differentiation, cell cycle, inflammation, and adverse environmental stimuli were shown to activate JNK signaling, although the biological outcome is often unknown (Kyriakis and Avruch, 2001; Weston and Davis, 2002). Deficiencies in JNK signaling have also been implicated in abnormal control of apoptosis. During embryonic development in *Drosophila*,

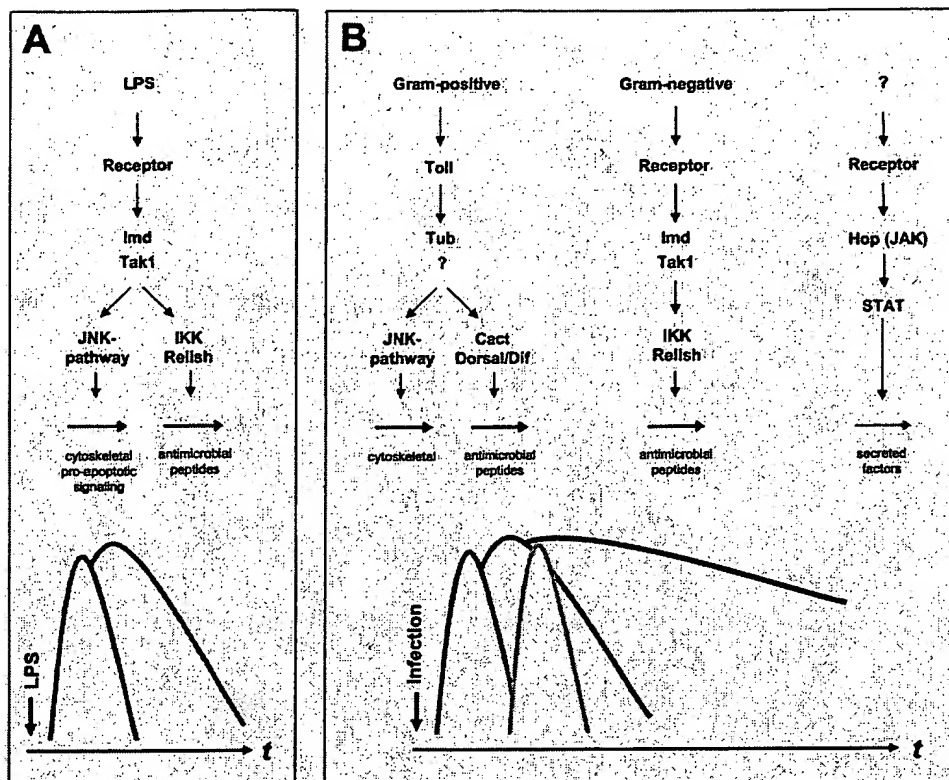


Figure 6. Model of Temporal Signaling Pathway Activation during Innate Immunity in *Drosophila*

(A) Signaling in SL2 cells in response to LPS.

(B) Signaling in adult *Drosophila* after septic injury. See text for details.

JNK signaling is required for the initiation of dorsal closure, a morphological process that requires epithelial sheet movements (reviewed in Stronach and Perrimon, 1999). We have shown here that JNK pathways act downstream of microbial stimuli, both in vivo and in cells, to induce cytoskeletal regulators. In SL2 cells, JNK signaling is required for the induction of a cluster of cytoskeletal, cell adhesion regulators and proapoptotic factors. Interestingly, both NF $\kappa$ B and JNK branches share the same upstream components, Tak1 and Imd, indicating that the activation of both processes are tightly linked. MMP-1, a matrix metalloproteinase that is one of the most markedly upregulated genes after LPS stimulation (Figure 1), has been implicated in wound-healing responses in mammals (reviewed in Raventi and Kahari, 2000). Compared with experiments in cells, the situation in vivo after septic injury is likely more complex. Gene expression profiling in whole organisms likely has a lower sensitivity for transcriptional changes that occur in rather small numbers of cells. Also, tissue-specific differences in signaling pathway activity may not reflect the transcriptional changes that we observe in our cell culture model. We found that muscle-specific cytoskeletal factors, possibly because we injected into the thoracic muscle, are not inducible in a JNK-deficient genetic background. However, since we needed to remove both Mkk4 and Hep (Mkk7) in cells to deplete JNK pathway activity, an experiment that we cannot perform in vivo because of the lack of an Mkk4 mutant, these experiments might not have uncovered all JNK-dependent

transcripts. SAPK/JNK modules can also be linked to different upstream activating cascades. For example, a recent study reported the activation of p38 $\alpha$  through a cascade involving Toll, TRAF6, and TAB (Ge et al., 2002). Similarly, during innate immune responses JNK pathways can be activated by both Toll and Imd pathways in vivo.

The activation of JNK signaling is reminiscent of signaling during dorsal and thorax closure (Stronach and Perrimon, 1999). In dorsal closure, SAPK/JNK signaling controls cytoskeletal rearrangements that lead to the epithelial sheet movements of the embryonic epidermis. In recent experiments, SAGE analysis of embryos with activated SAPK/JNK signaling show an induction of cytoskeletal factors (Jasper et al., 2001). Also, dorsal closure movements were proposed to be similar to the reepithelization that occurs during wound healing (Jacinto et al., 2001). In other developmental contexts, SAPK/JNK signaling has been implicated in cytoskeletal rearrangements and cell motility, such as the generation of planar polarity in *Drosophila* and convergent-extension movements in vertebrates (Boutros et al., 1998; Yamanaka et al., 2002). A common theme of SAPK/JNK pathways might be their control of cytoskeletal regulators for diverse biological processes. Our finding that, in response to LPS, SAPK/JNK and NF $\kappa$ B targets are coregulated through the same intracellular pathway suggests a close linkage of directed antimicrobial activities and tissue repair processes.

In conclusion, we employed genome-wide expression

profiling to examine the contribution of different signaling pathways in complex tissues and to assign targets to candidate pathways. We have used both a cell culture model system and an in vivo analysis to show the temporal order of NF $\kappa$ B-dependent and -independent pathways after septic injury. An interesting question that remains is, how do the extracellular events leading to pathway activation reflect the nature of the pathogen? We noticed that clean injury experiments induced a largely overlapping set of induced genes, but to a lower extent than septic injury (data not shown). This is consistent with experiments showing that septic injury with only gram-negative *E. coli* induced both anti-gram-negative and anti-gram-positive responses (data not shown; Irving et al., 2001). Our interpretation of these results is that wounding, in itself, might be sufficient to induce a transient (and unspecific) innate immune response. However, further studies are needed to understand the nature of the inducing agent.

Analysis of signaling pathway signatures could be a useful undertaking in order to rapidly dissect complex biological processes into smaller functional modules, such as signaling cassettes that are linked to specific transcriptional responses. The analysis of signaling pathways in various biological contexts may lead to the identification of "functional signatures" such as the proposed role of JNK pathways in cytoskeletal regulation. One of the key challenges in the future will be to integrate spatial and temporal analysis of signaling pathways to build models that reflect the complexity of signaling in vivo. We propose that the biological response after microbial infection consists of a "module" that leads to the expression of antimicrobial peptides and a second module that controls cytoskeletal gene expression, reminiscent of epithelial movement and wound-healing processes. Both processes might be coupled through a common upstream signaling pathway because infections often occur through a breach of epithelial structures.

#### Experimental Procedures

##### *Drosophila* Strains and Septic Injury Experiments

All flies were reared at 23°C, unless otherwise noted. Oregon R was used for wild-type experiments. The following mutant *Drosophila* strains were used in the described experiments: *tub<sup>118</sup>/tub<sup>238</sup>*, *cact<sup>639</sup>/cact<sup>42</sup>*, *key<sup>1</sup>/key<sup>1</sup>*, *rel<sup>E20</sup>/rel<sup>E20</sup>*, *T1<sup>10</sup>*, *hop<sup>mar1</sup>/hop<sup>MS8</sup>*, and *hep<sup>1</sup>* (Hedengren et al., 1999; Lemaître et al., 1996; Lu et al., 2001). Microbial infections with *E. coli* and *M. luteus* were done as previously described (Lemaître et al., 1995b). In brief, a concentrated mix of gram-positive and gram-negative bacteria was obtained by pooling overnight cultures of *E. coli* and *M. luteus*. Bacterial cultures were pelleted and resuspended in one-tenth of the original volume prior to infection experiments. A thin tungsten needle (Fulham) was loaded by dipping into gram-negative/gram-positive bacteria cocktail, and septic injury experiments were performed on 5- to 7-day-old adult *Drosophila* by pricking them in the thoracic segments. At indicated time points, *Drosophila* were snap-frozen in liquid nitrogen and stored at -70°C prior to further processing.

##### Cell Culture and dsRNA Experiments

The LPS-responsive Schneider SL2 cell line (a gift from Katja Brueckner) was cultured in Schneider's medium (GibcoBRL) supplemented with 10% fetal bovine serum (JRH Bioscience) and Penicillin-Streptomycin (GibcoBRL) at 25°C, unless noted otherwise. One day before the addition of dsRNA, 10<sup>6</sup> cells were seeded in 3 cm cell culture dishes (Falcon). The next day, cells were washed twice

in DES medium (Sigma) without serum before treatment with 15  $\mu$ g dsRNA in 1 ml DES medium. After 45 min, 2 ml of serum-supplemented Schneider's medium was added to each well, and cells were incubated for a further 72 hr to ensure protein depletion. Cells were then used in LPS (*E. coli* strain O55:B5 [Sigma], 10  $\mu$ g/ml) stimulation and control experiments. dsRNA was generated and used essentially as previously described (Clemens et al., 2000). Briefly, gene-specific primers were designed to include T7 promoter sequences and were subsequently used to amplify fragments by PCR from genomic DNA. The PCR product was used in in vitro transcription (T7 MEGAscript Kit; Ambion) to generate both RNA strands in one reaction. RNA was purified with RNeasy columns (Qiagen) and quality controlled by agarose gel electrophoresis. For expression profiling experiments, we pooled three 3 cm cell culture experiments for each labeling reaction. Total RNA was isolated by Trizol according to the manufacturer's specifications (GibcoBRL). At least two labeling reactions and hybridizations were performed for each experimental condition.

##### Oligonucleotide Microarray Hybridization

Reverse transcription and biotin labeling of 20  $\mu$ g total RNA was done as described before (Wodicka et al., 1997). Twenty micrograms of cRNA was fragmented and hybridized for 16 hr at 45°C to *Drosophila* Genome GeneChips (Affymetrix), representing probes for over 13,600 known and predicted open reading frames. Microarrays were washed, stained, and scanned with a PMT-adjusted Agilent scanner according to standard GeneChip protocols (Affymetrix). At least two total RNA samples were independently labeled and used for separate GeneChip hybridizations.

##### Computational Analysis

Microarray Suite 4.0 (Affymetrix) was used for raw intensity value calculations. Intensity value files (CEL) were used as input for dChip (Li and Wong, 2001) to calculate model-based expression levels. Data sets were rejected when array probe or single probe outliers exceeded 1.5%. Following a per array quality control, intensity data was scaled by smoothing-spline normalization, and absolute expression values for each array were calculated with a model-based algorithm (Li and Wong, 2001). Low and negative expression values were truncated to 10. dChip probe-sensitivity indices were handled separately for adult *Drosophila* and cell culture specimen. Data sets from replicate arrays were merged after calculation of model-based expression levels. Genes were filtered by criteria that require a 90% confidence interval of a 1.4-fold change. In addition, we required that the difference in expression levels to be greater than 50. For the adult time-course analysis, we required that, to be selected, a gene must be at least twice the indicated threshold. Filtered genes for the 6 hr time point and mutant analysis have a minimum difference in expression levels of 100. Hierarchical clustering was performed with a Euclidean distance measure. To search for pattern similarities in pathway mutants, we calculated similarities by  $c_i = 1/\sqrt{n} \sum (x_i - \mu_x)(sp_i - \mu_{sp})/\sigma_x \sigma_{sp}$ , where  $x$  is a vector of expression levels and  $sp$  is a vector representing the search pattern (Table 1).

##### Quantitative RT PCR

Quantitative PCR was performed according to the manufacturer's instructions. In brief, for each experiment, total RNA was isolated by Trizol (GibcoBRL) extraction from two 3 cm cell dishes. Ten micrograms of total RNA was treated for 30 min with DNase I prior to a 2 hr reverse transcription reaction by Superscript RT II (GibcoBRL), following the manufacturer's instructions. Quantitative PCR was performed with a LightCycler PCR machine and FastStart SYBR Green Kit (Roche). Melting curves were used as quality controls to exclude samples with genomic DNA content and to ensure single-product amplification. *rp49* levels were used as internal normalization controls. Data was analyzed with the LightCycler 3.0 software package (Roche).

##### Acknowledgments

We would like to thank Dominique Ferrandon, Dan Hultmark, Bruno Lemaître, and David Schneider for fly strains. We are grateful to the Biopolymer Facility for use of their GeneChip workstation. We thank



Marc Halfon, Bernard Mathey-Prevot, Henri Jasper, Dirk Bohmann, and Lutz Kockel for critical comments on the manuscript. H.A. is a Research Associate at the Institut National de la Recherche Agonomique. M.B. was supported by an Emmy-Noether fellowship from the Deutsche Forschungsgemeinschaft. N.P. is an Investigator of the Howard Hughes Medical Institute.

Received: March 6, 2002

Revised: September 19, 2002

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# Targeting of TAK1 by the NF- $\kappa$ B protein Relish regulates the JNK-mediated immune response in *Drosophila*

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The molecular circuitry underlying innate immunity is constructed of multiple, evolutionarily conserved signaling modules with distinct regulatory targets. The MAP kinases and the IKK-NF- $\kappa$ B molecules play important roles in the initiation of immune effector responses. We have found that the *Drosophila* NF- $\kappa$ B protein Relish plays a crucial role in limiting the duration of JNK activation and output in response to Gram-negative infections. Relish activation is linked to proteasomal degradation of TAK1, the upstream MAP kinase kinase kinase required for JNK activation. Degradation of TAK1 leads to a rapid termination of JNK signaling, resulting in a transient JNK-dependent response that precedes the sustained induction of Relish-dependent innate immune loci. Because the IKK-NF- $\kappa$ B module also negatively regulates JNK activation in mammals, thereby controlling inflammation-induced apoptosis, the regulatory cross-talk between the JNK and NF- $\kappa$ B pathways appears to be broadly conserved.

[Keywords: MAPKKK; signal transduction; rel protein; proteasome; Imd]

Received November 6, 2003; revised version accepted January 28, 2004.

Multicellular organisms defend themselves from microbial infections by mounting an innate immune response that serves to neutralize pathogenic invaders (Kimbrell and Beutler 2001; Janeway and Medzhitov 2002). Receptors that recognize pathogen-associated molecular patterns (PAMPs) trigger signaling cascades that culminate in expression of immune effector genes encoding antimicrobial peptides, cytokines, chemokines, and various inflammatory mediators. In both vertebrates and invertebrates, the NF- $\kappa$ B family of transcription factors serves a pivotal role in signal-induced gene activation during the innate immune response (Silverman and Maniatis 2001).

Owing to the broad evolutionary conservation of innate immunity, studies in genetically tractable invertebrates, especially *Drosophila melanogaster*, have contributed greatly to our understanding of the mechanisms underlying innate immunity. *Drosophila*, like other invertebrates, lacks an adaptive immune system, being entirely dependent on innate immunity for its resistance to microbial infection. Genetic screens for *Drosophila* mu-

tants susceptible to pathogenic challenge have identified two distinct signaling cascades—the Toll and Imd pathways—that govern expression of antimicrobial peptides (Hoffmann and Reichhart 2002; Tzou et al. 2002; Hultmark 2003).

The Toll pathway, originally characterized for its role in establishing embryonic dorsoventral polarity, mediates resistance to fungal and Gram-positive infections by activating two NF- $\kappa$ B proteins: Dorsal and Dif (Wasserman 2000). The Imd pathway induces expression of a distinct set of genes coding for antimicrobial peptides, such as Diptericin and Attacin, that provide immunity to Gram-negative bacteria. Components of the Imd pathway include a peptidoglycan recognition protein (PCRPLC; Choe et al. 2002; Gottar et al. 2002; Ramet et al. 2002), as well as several counterparts to signal transducers involved in NF- $\kappa$ B activation in mammalian cells. These include IMD itself, a homolog of the TNF signaling pathway kinase RIP (Georgel et al. 2001), as well as TAK1, a MAP kinase kinase kinase (MAPKKK), and two I $\kappa$ B kinase (IKK) complex components, IKK $\beta$  (ird5), and IKK $\gamma$  (Kenny; Rutschmann et al. 2000; Silverman et al. 2000, 2003; Lu et al. 2001; Vidal et al. 2001). In conjunction with the IKK complex, the *Drosophila* caspase Dredd functions in proteolytic activation of a third NF- $\kappa$ B family protein, Relish, that directly activates antibacte-

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1168104>.

rial peptide genes (Leulier et al. 2000; Stöven et al. 2000, 2003). There is also evidence that the *Drosophila* FADD homolog acts downstream of IMD, independently of TAK1 and IKK, to control Dredd activation (Leulier et al. 2002; Naitza et al. 2002).

Although Relish, Dif, and Dorsal are critical for transcriptional induction of antimicrobial peptides, they are unlikely to be the sole mediators of PAMP-triggered signal transduction. In mammalian macrophages and dendritic cells, Toll-like receptors (TLRs) specific to particular PAMP ligands induce activation of ERK, JNK, and p38 MAP kinases (MAPKs) in addition to NF- $\kappa$ B (Akira et al. 2001). Furthermore, the nematode *Caenorhabditis elegans* and the plant *Arabidopsis thaliana* encode no orthologs of NF- $\kappa$ B, but rely instead on MAPK signaling cascades to mediate innate immune responses (Asai et al. 2002; Kim et al. 2002; Mallo et al. 2002).

JNK signaling is required for a variety of biological processes in flies and mammals, including developmental morphogenesis, apoptosis, and immunity (Stronach and Perrimon 1999; Chang and Karin 2001). In mammals, JNK activation is required for inflammation-induced apoptosis (Deng et al. 2003; Maeda et al. 2003). Curiously, JNK activation is negatively regulated by the IKK-NF- $\kappa$ B pathway (De Smaele et al. 2001; Tang et al. 2001). Although this cross-talk appears to be an important mediator of the antiapoptotic function of NF- $\kappa$ B (Karin and Lin 2002), the mechanism by which NF- $\kappa$ B negatively regulates JNK activation in mammals is not well understood (Kyriakis 2001). In *Drosophila*, lipopolysaccharide (LPS) has been reported to activate JNK (Sluss et al. 1996), which is required for induction of a subset of immune response genes (Boutros et al. 2002), as well as the NF- $\kappa$ B family member Relish, which directs transcription of a distinct group of immune response loci (Boutros et al. 2002). However, it has been difficult to assess the biological role of the JNK module in the innate immune response because loss-of-function mutations in this pathway are usually embryonic lethal (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996).

We have found that activation of the Relish module in *Drosophila* results in attenuation of JNK activity after challenge with a commercial LPS preparation. Moreover, we demonstrate that Relish activation is mechanistically linked to switching off the JNK signaling module after LPS stimulation. In the absence of Relish activation, LPS treatment causes a sustained activation of JNK, which, in turn, leads to prolonged expression of JNK target genes. Biochemical experiments reveal that the negative regulation of JNK signaling involves proteasomal degradation of TAK1. Hence, it appears that the Relish-dependent genes not only serve their own immune effector roles, but also participate in shaping the temporal pattern of the JNK-mediated immune response. These findings reveal an unanticipated cross-talk between *Drosophila* innate immune pathways and hold promise for mechanistic insight into the mammalian counterpart of this regulatory interaction.

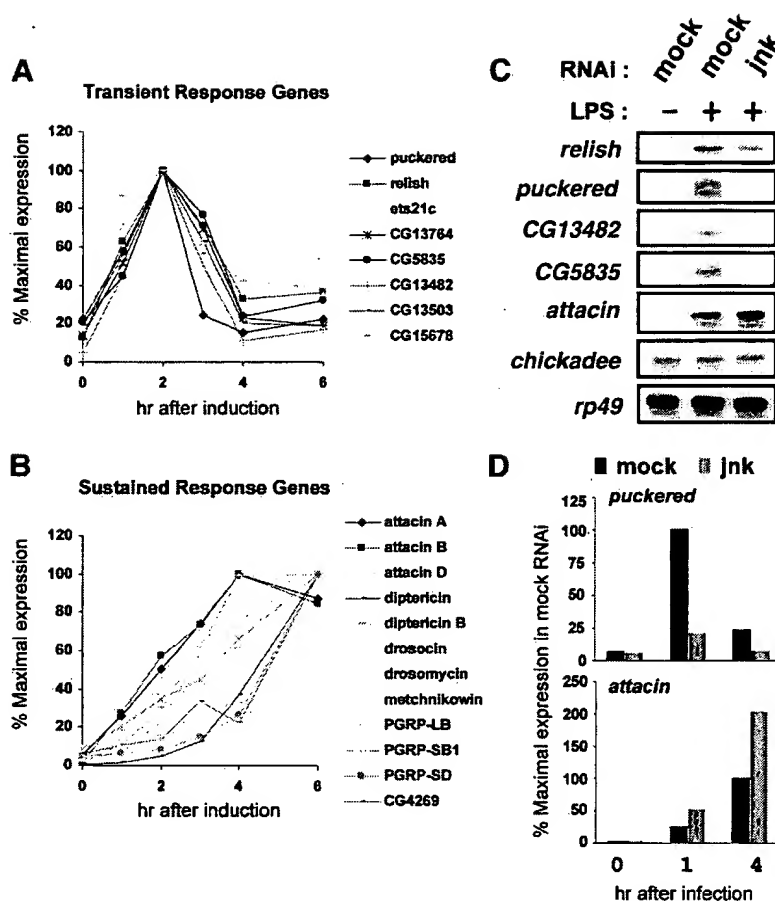
## Results

### *An LPS preparation induces transient and sustained transcriptional responses via distinct signaling modules*

Activation of innate immune signaling in whole flies can arise from the primary challenge with the infectious organism and from secondary responses to cytokines and inflammatory mediators produced during the primary response. To reduce the complexity of the systemic immune response, we chose to work with cultured S2 cells. These cells are known to be immune-responsive and are believed to be derived from phagocytes (Ramet et al. 2002). We first pretreated the cells with ecdysone, which enhances the innate immune response (Dimarcq et al. 1997). Then, 1 d after ecdysone treatment, we exposed the cells to a commercial LPS preparation; such preparations contain Gram-negative bacterial peptidoglycan that induces the Imd pathway (Leulier et al. 2003). We then isolated total RNA at different time points post-challenge and analyzed the mRNA expression profile using high-density oligonucleotide microarrays representing nearly all of the *Drosophila* transcriptome. To identify genes exhibiting the largest response, we screened for loci with an increase in mRNA expression of at least fourfold within 6 h of treatment. In all, 64 genes met this criterion. We then restricted our analysis to a subset of 27 genes that were induced at least twofold within 90 min of bacterial infection of adult flies, based on published data obtained with the same array platform and data analysis scheme (De Gregorio et al. 2001).

The responsive genes could be readily divided into two classes (Fig. 1A,B). Expression of transient response genes peaked at 2 h and declined two- to ninefold between the 2- and 4-h time points. In contrast, expression of the sustained response genes remained steady (less than twofold variation) or increased from 2 h of LPS addition onward. The composition of the two response classes was markedly different. Most of the sustained response genes encoded either antimicrobial peptides or peptidoglycan recognition proteins (Fig. 1A) and belonged to the group of Relish-dependent genes (De Gregorio et al. 2002). Neither type of protein was found among the products of the transient response class, which, instead, contained several novel genes that had not previously been classified as immune response loci (Fig. 1B; data not shown). The fact that their expression very quickly returned to uninduced levels precluded their passing the screening criteria previously used in identifying immune response loci in whole flies (De Gregorio et al. 2002). Many of the same transient response genes were, however, identified in an analogous study carried out without steroid hormone treatment (Boutros et al. 2002).

As induction of many transient response genes by LPS preparations, including *puckered*, requires JNK signaling (Boutros et al. 2002), we treated S2 cells with JNK-specific double-stranded RNA to deplete endogenous JNK by RNA interference (RNAi). The cells were then challenged with LPS, and their gene expression profile analyzed by an RNase protection assay (Fig. 1C) and by real-



**Figure 1.** Requirement for JNK in the transient response gene induction by LPS. (A) LPS-induced transient response gene expression in S2 cells. S2 cells were stimulated with LPS for the length of time indicated on the abscissa. Total RNA was prepared and analyzed using the Affymetrix *Drosophila* gene chip. Values on the ordinate indicate the relative mRNA level. (B) LPS-induced sustained response gene expression in S2 cells. (C) S2 cells were mock-treated or treated with jnk dsRNA for 3 d and then left unstimulated or stimulated with LPS for 1 h. Total RNA isolated from those cells was analyzed by RNase protection to assay the expression of the genes listed on the left. In this and subsequent panels, *attacin* refers to the *attacin A* gene. (D) After treating S2 cells with dsRNA and LPS as in C, the *puckered* and *attacin* mRNA levels in S2 cells were measured by real-time PCR analysis. The results shown are averages of three determinations.

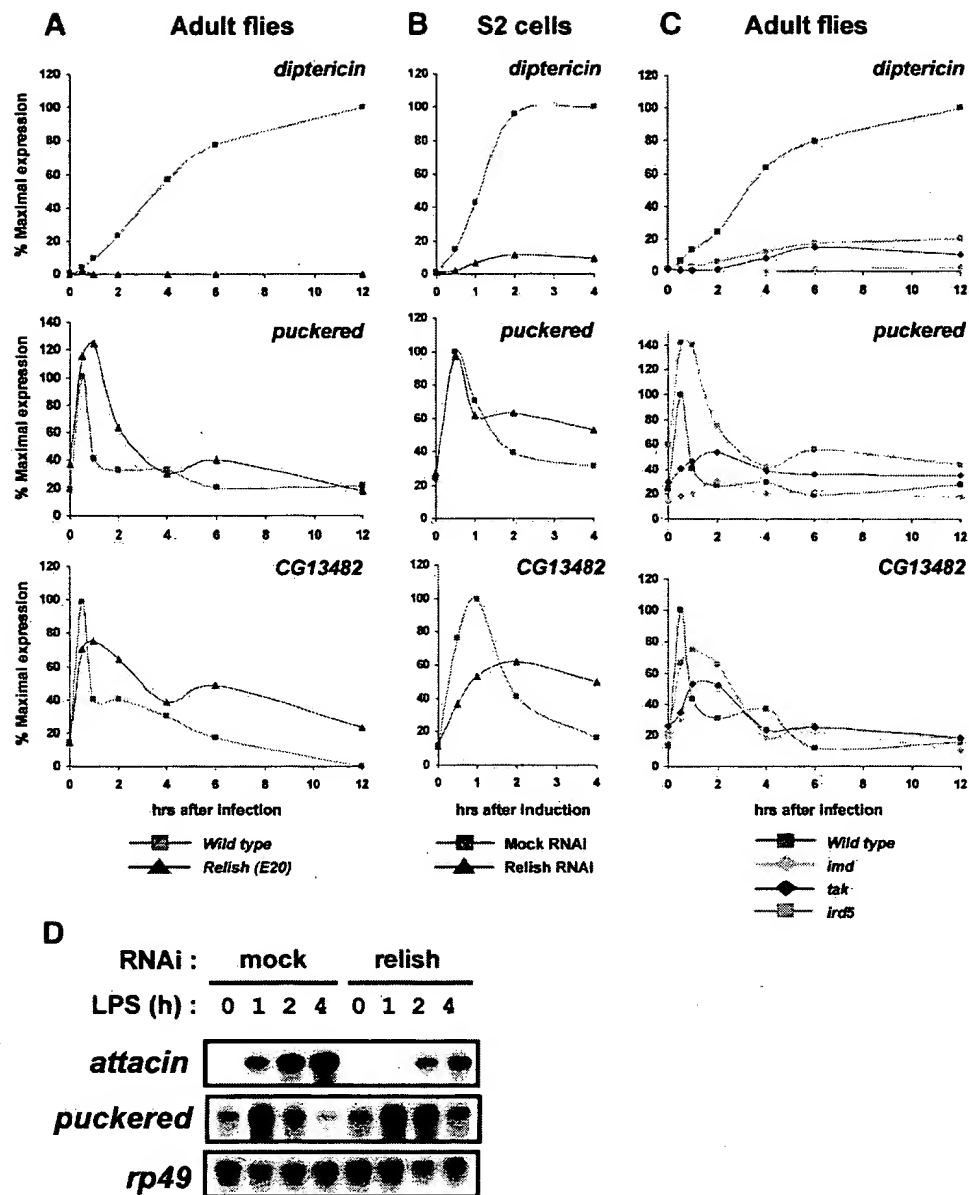
time PCR analysis (Fig. 1D). When we examined expression of four transient response genes (*Relish*, *puckered*, *CG13482*, and *CG5835*), we found that their induced expression was greatly diminished or abolished by JNK dsRNA treatment. In contrast, expression of the *Relish* target gene *attacin* was not abolished and, indeed, increased slightly, upon JNK inactivation by RNAi. This finding, together with previous results (Boutros et al. 2002), indicates that JNK signaling governs expression of transient response genes in *Drosophila* immunity. Furthermore, the JNK and *Relish* branches of the Imd pathway control induction of genes that differ with regard to their temporal patterns of expression and their function.

#### Defects in the IKK/*Relish* module result in prolonged expression of JNK target genes

In infected flies, cytokines (Agaisse et al. 2003) and other inflammatory mediators (Foley and O'Farrell 2003) mediate secondary responses that have the potential to contribute to JNK activation. Temporal expression patterns and requirements for particular signaling modules could therefore differ from those in S2 cells. To address this possibility, we analyzed expression of transient and sustained response genes in wild-type (wt) flies.

Infection of wild-type flies with the Gram-negative bacterium *Enterobacter cloacae* induced JNK target gene expression with a pattern similar to that observed in LPS-treated S2 cells (Fig. 2A). The *puckered* and *CG13482* loci were induced immediately after infection, with expression peaking after 30 min and then declining rapidly to basal levels. Other transient response genes also exhibited similar patterns of induced expression in both cells and flies (data not shown). In contrast, *dipteracin* expression in wild-type flies increased gradually and continued to increase until 12 h of infection. Thus, the two temporal patterns of induction seen in S2 cells also characterize the immune response in whole flies.

In the Imd pathway, signaling branches downstream from TAK1 (Boutros et al. 2002; Chen et al. 2002). The JNK signaling cascade is required for expression of transient response genes, whereas the IKK/*Relish* module is essential for sustained gene induction (Boutros et al. 2002). To determine whether there is any cross-talk between these two branches of the Imd pathway, we repeated the infection experiments, using adult flies mutant for individual pathway components. Evidence for cross-talk was readily apparent in studies with *relish* mutant flies. Although *Relish* was not required for induction of JNK target genes, the *relish* E20 mutation increased the duration (*puckered* and *CG13482*) and in



**Figure 2.** Prolonged expression of JNK targets in flies and S2 cells that have a defect in Relish activation. (A) Wild-type and *relish* mutant adult flies were infected by wounding with a needle dipped in *E. cloacae*. Total RNA was prepared at the indicated time points after infection and subjected to real-time PCR analysis. (B) S2 cells were mock-treated or treated with *relish* dsRNAs for 3 d and then left unstimulated or stimulated with LPS. Total RNA was prepared and analyzed as in A. (C) Wild-type adult flies and *imd*, *tak1*, and *ird5* mutant flies were analyzed as in A. (D) RNAi in S2 cells and LPS stimulation were carried out as in B. Total RNA was analyzed by RNase protection assay to measure the expression of the genes listed on the left.

some cases the intensity (*puckered*) of target gene expression. These effects did not reflect residual Relish activity or any peculiarity of the E20 mutation, because *dipterocin* expression was abolished (Fig. 2A) and the prolonged expression was also seen in a *relish* mutant line harboring a different mutation (E38 allele; Hedengren et al. 1999; data not shown). Furthermore, similar effects were observed in S2 cells treated with ds *relish* RNA. For example, LPS-induced *attacin* expression was greatly reduced, whereas both *puckered* and *CG13482* displayed

sustained expression (Fig. 2B,D). Thus, Relish acts as a negative regulator of JNK target gene expression in both flies and cultured cells.

To determine the point of intersection between the branches of the Imd pathway, we extended our studies to mutations in other pathway components. In *ird5* flies, JNK target gene expression exhibited a broadened peak of induction, whereas *dipterocin* expression was substantially decreased, a pattern very similar to that in the *relish* mutants (Fig. 2C). In contrast, flies mutant for ei-

ther *imd* or *tak1* were incapable of activating either class of genes, indicating that these loci are essential for triggering both the IKK/Relish and the JNK signaling cascades. We noted, however, that the residual induction of JNK targets in *imd* and *tak1* mutant flies also followed a broadened time course.

#### *Inhibition of Relish activation results in a sustained JNK activation after challenge*

To explore the mechanism of Relish-mediated negative regulation of JNK target genes, we used RNAi to deplete S2 cells either of Relish or of Ird5 or Kenny, components of the IKK complex that directs proteolytic activation of Relish. We then monitored JNK activation in the treated cells by immunoblot analysis, using the phospho-JNK-specific antibody that detects the activated enzyme. In mock-treated cells, phosphorylated JNK was detected at 15 and 30 min after LPS stimulation, with its levels diminishing almost to that of the unstimulated state by 45 min (Fig. 3A). In contrast, RNAi against *kenny*, *ird5*, or *relish* resulted in JNK activation that persisted for at least 60 min after LPS stimulation. We conclude that Relish processing is required for transient JNK activation by the Imd pathway.

To determine whether Relish-mediated target gene expression is required for JNK regulation, we examined whether down-regulation of JNK in the LPS response requires ongoing RNA and protein synthesis. Pretreatment of S2 cells with either the transcriptional inhibitor actinomycin D or the translational inhibitor cycloheximide resulted in a sustained JNK activation upon LPS stimulation (Fig. 3B). These inhibitors also blocked the resynthesis of full-length Relish protein following proteolytic depletion of the pool present prior to LPS stimulation.

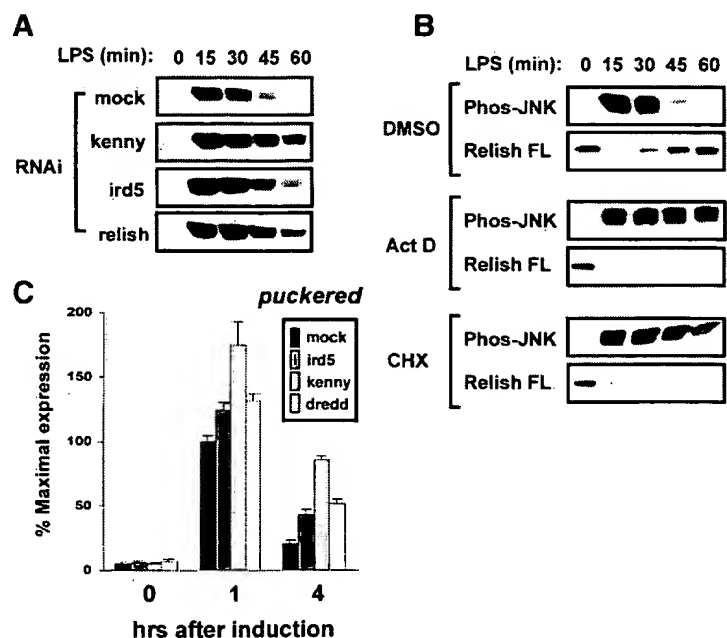
These results suggest that activation of one or more target genes by processed Relish protein is required for rapid shutoff of JNK activity. The Relish-dependent negative regulation of JNK signaling is distinct from the previously described negative feedback loop involving the MAPK phosphatase Puckered (Martin-Blanco et al. 1998), because *puckered* expression is enhanced, not suppressed, in response to inhibition of Relish (see Fig. 2).

To examine whether a prolonged JNK activation in S2 cells lacking Relish activation affects JNK target expression, the level of *puckered* mRNA was measured after RNAi against *ird5*, *kenny*, and *dredd*. Like S2 cells devoid of Relish itself (Fig. 2B), S2 cells depleted of the components in either branch of the Imd pathway showed an increased and prolonged expression of *puckered* (Fig. 3C).

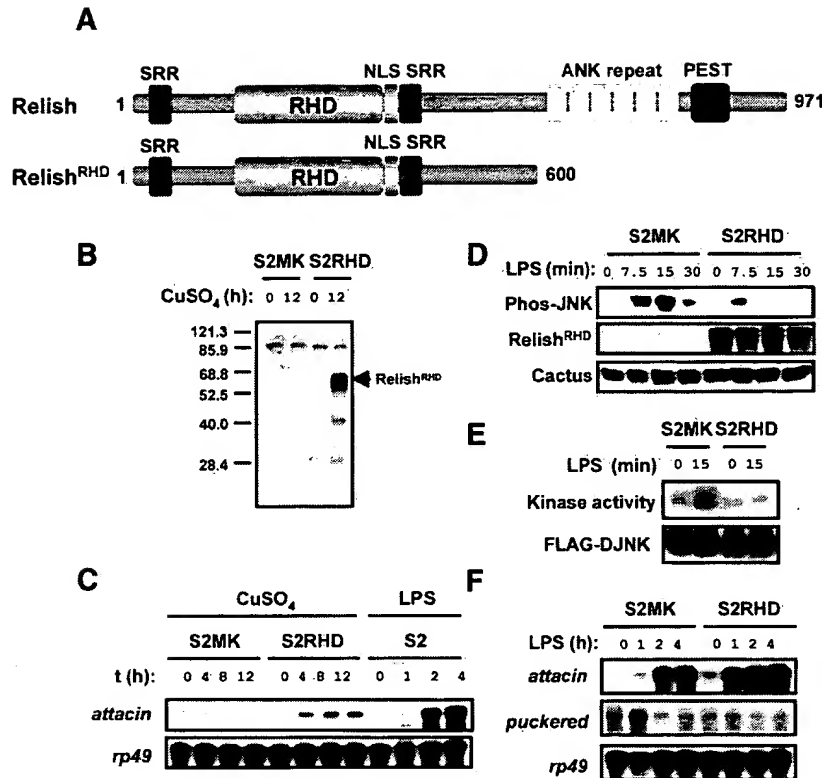
#### *Relish activation prior to Imd pathway activation attenuates JNK activation*

The notion that Relish is responsible for preventing sustained JNK activation implies that there is a step in the JNK signaling cascade that is sensitive to the inhibitory action of Relish or one of its targets. To test this hypothesis, we devised a protocol in which we temporally separated activation of Relish from that of JNK and then examined whether forced Relish activation prior to LPS stimulation suppressed JNK activation. To this end, we used a constitutively active Relish that contains the N-terminal 600 amino acids, including the Rel-homology domain (Dushay et al. 1996). This derivative, designated RHD, is similar to mature nuclear Relish, which is generated by endoproteolytic cleavage (Fig. 4A; Stöven et al. 2000). We generated an S2 cell line stably transfected with a copper-inducible RHD construct (S2RHD), as well

**Figure 3.** Sustained activation of JNK signaling in S2 cells lacking Relish activation. (A) S2 cells were treated with dsRNAs specific to *kenny*, *ird5*, or *relish* for 3 d and then unstimulated or stimulated with LPS for the length of time indicated. Cell lysates were prepared and analyzed by immunoblotting with antibodies specific to the phosphorylated forms of JNK. (B) S2 cells were treated with 0.1% (v/v) dimethylsulfoxide (DMSO), 1  $\mu$ g/mL actinomycin D (Act D), or 25  $\mu$ g/mL cycloheximide (CHX) for 1 h and then unstimulated or stimulated with LPS for the length of time indicated. Cell lysates were prepared and analyzed as in A with antibodies specific to the phosphorylated forms of JNK (Phos-JNK) and Relish. For Relish, only the full-length (FL) form is shown. (C) S2 cells were mock-treated or were treated with *ird5*, *kenny*, or *dredd* dsRNAs and analyzed as in Figure 2B.







**Figure 4.** Inhibition of the JNK signaling module by Relish activation prior to LPS stimulation. (A) Structure of Relish protein and its Rel-homology domain (RHD) derivative. (B) S2 cells stably transfected with this RHD construct (S2RHD) and control cells transfected with the backbone vector construct (S2MK) were untreated or treated with 0.7 mM CuSO<sub>4</sub> for 12 h. Cell lysates were prepared and analyzed by immunoblotting with antibodies specific to the RHD of Relish (Relish<sup>RHD</sup>). (C) S2MK and S2RHD cells were unstimulated or stimulated with CuSO<sub>4</sub>. Total RNA was prepared after the length of time indicated and analyzed by RNase protection assay. (D) S2MK and S2RHD cells were treated with CuSO<sub>4</sub> for 12 h and then unstimulated or stimulated with LPS for the length of time indicated. Cell lysates were prepared and analyzed by immunoblotting with antibodies specific to phos-JNK, Relish<sup>RHD</sup>, and Cactus. (E) S2MK and S2RHD cells were transfected with a plasmid that expresses Flag-DJNK and then treated with CuSO<sub>4</sub> and stimulated with LPS as in D. Flag-DJNK proteins were immunoprecipitated from cell lysates and subjected to *in vitro* kinase assay using GST-DJun as a substrate. (F) S2MK and S2RHD cells were treated with CuSO<sub>4</sub> and stimulated with LPS. Total RNA isolated at the indicated time point was analyzed by RNase protection assay.

as a control cell line with the backbone vector construct (S2MK). By immunoblot analysis, we confirmed high-level expression of RHD in induced S2RHD cells (Fig. 4B). We also confirmed that the expressed RHD protein was functional by examining Relish target gene expression. Induction of RHD expression directed *attacin* expression, albeit with a lower efficiency than LPS treatment (Fig. 4C). Other Relish targets were induced to a similar extent by RHD, whereas JNK targets did not show any noticeable induction (data not shown).

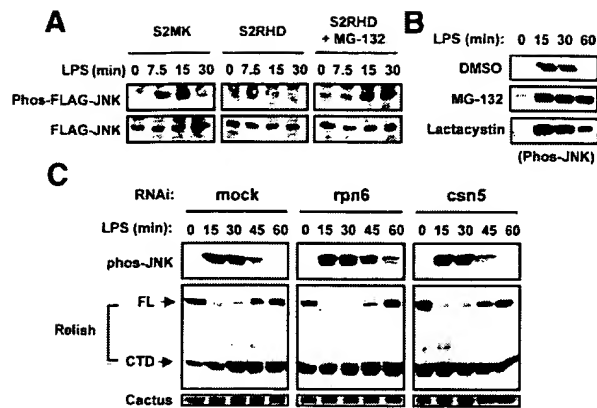
Next we tested whether RHD expression prior to Imd pathway activation could inhibit JNK signaling. For this, 12 h after copper addition, we stimulated cells with LPS and then examined JNK activation by two methods. First, we analyzed the level of phosphorylated JNK in the protein extract by immunoblotting. JNK activation in S2RHD cells was greatly reduced in level and duration in comparison with the S2MK line (Fig. 4D). In a second set of experiments, we transfected S2MK and S2RHD with an expression vector for Flag-epitope-tagged JNK and then performed an immune complex kinase assay. We found that RHD expression almost completely inhibited activation of JNK in response to LPS (Fig. 4E). Importantly, this effect was not due to reduced JNK expression. Consistent with our model for Relish function, the inhibition of JNK signaling by RHD expression also altered the induction of target genes. In copper-treated S2RHD cells, *puckered* induction was abolished, whereas the onset of

*attacin* induction was shifted toward an earlier time point (Fig. 4F).

#### Relish-mediated inactivation of JNK signaling requires target protein degradation

To further investigate the mechanism underlying Relish-mediated JNK inactivation, we treated S2 cells with an array of chemical agents that perturb inflammatory processes in mammalian immune cells, screening for any that relieved the RHD-mediated inhibition of JNK activation. The compounds analyzed included inhibitors to different classes of serine proteases, cathepsins and caspases, proteasomal inhibitors, scavengers of reactive oxygen and nitrogen intermediates, alkalinizing agents, nitric oxide synthase inhibitors, and NADPH oxidase inhibitors.

Of the compounds tested, only the proteasomal inhibitors MG-132 and lactacystin were effective in restoring LPS-induced JNK activation in copper-treated S2RHD cells (Fig. 5A; data not shown). To confirm that the effect of these inhibitors on JNK activation reflected a restoration of signaling rather than a stabilization of JNK, we transfected Flag-JNK into S2RHD cells and examined the levels of total and phosphorylated JNK. As shown in Figure 5A, MG-132 increased JNK activation without affecting the amount of JNK protein, indicating that MG-132 inhibits a Relish-dependent proteolytic event re-



**Figure 5.** Involvement of proteasomal degradation in Relish-dependent JNK inhibition. (A) S2MK and S2RHD cells were transfected with Flag-DJNK expression plasmid and then treated with  $\text{CuSO}_4$ . S2RHD cells were untreated or further treated with 50  $\mu\text{M}$  MG-132 for 2 h. After stimulating with LPS, cell lysates were prepared and analyzed by immunoblotting with antibodies specific to phos-JNK (upper panels) and Flag (lower panels). (B) S2 cells were treated with DMSO, 50  $\mu\text{M}$  MG-132, or 5  $\mu\text{M}$  lactacystin for 2 h and then unstimulated or stimulated with LPS for the length of time indicated. Phos-JNK was analyzed by immunoblotting. (C) S2 cells were treated with dsRNAs specific to *rpn6* or *csn5* for 3 d and then unstimulated or stimulated with LPS for the length of time indicated. Cell lysates were prepared and analyzed by immunoblotting with the antibodies indicated on the left.

sponsible for attenuation of JNK activation. We also confirmed that proteasomal degradation is essential for negative feedback regulation of JNK during the LPS response in untransfected S2 cells. Pretreatment with MG-132 or lactacystin resulted in sustained JNK activation (Fig. 5B) in much the same way as did RNAi against IKK or Relish or pretreatment with actinomycin D or cycloheximide (see Fig. 3A,B).

To confirm a requirement for the proteasome in JNK inactivation during the LPS response, we used RNAi in S2 cells to inactivate Rpn6, a component of the 19S proteasomal regulatory complex. As a control, we also used RNAi against Csn5/Jab1, a component of the nonproteasomal COP9 protein degradation complex. RNAi against Rpn6, but not Csn5, promoted sustained JNK activation (Fig. 5C). Under the same conditions, processing of Relish, as assayed by levels of full-length protein, was unaffected. Because inactivation of SkpA and Slimb, components of the SCF-E3 ubiquitin ligase, has been shown to increase the level of both full-length and processed Relish protein (Khush et al. 2002), we also carried out RNAi against these factors. RNAi against SkpA or Slimb had no effect on JNK activation (data not shown). We conclude that a ubiquitin E3 ligase other than SCF-E3 mediates the negative regulation of JNK activation.

#### *TAK1 is the target for Relish-mediated feedback regulation*

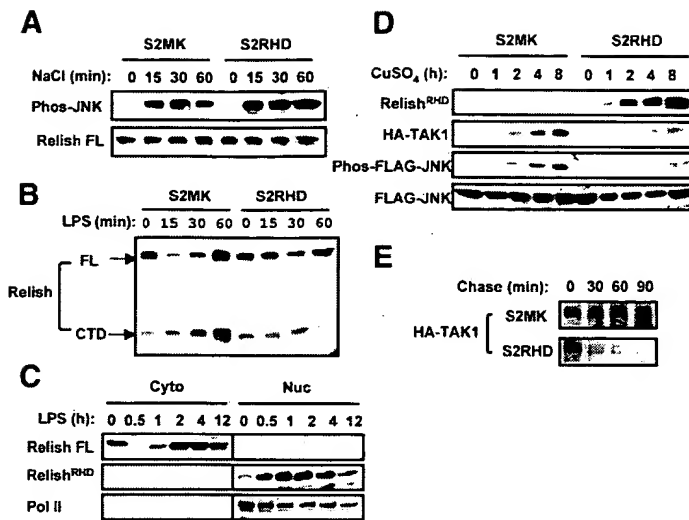
To identify the target for proteasome-mediated inhibition of JNK activation, we first examined whether such

inhibition is specific to the Imd pathway. Among other stimuli known to activate JNK in *Drosophila*, we chose to examine hyperosmolarity. Multiple MAPKKs, including TAK1, Slipper, Ask, and Mek1, mediate JNK activation in S2 cells subjected to osmotic stress triggered by sorbitol or NaCl (Chen et al. 2002). In the case of MAPKKs, however, osmotic stress activates the same enzymes, Hep/Mkk7 and Mkk4, as does LPS. In control S2MK cells, we observed JNK activation, but not Relish processing, within 15 min after addition of NaCl to the culture medium; this activation persisted for at least 60 min (Fig. 6A). We detected similar activation of JNK by NaCl in copper-treated S2RHD cells, even when RHD was induced 12 h prior to osmotic stress. Because such RHD expression suppressed JNK activation by LPS (see Fig. 4D), these results suggest that Relish does not inhibit JNK directly, nor does it target the two MAPKKs common to the LPS and osmotic stress pathways.

We next examined the effect of RHD overexpression on the LPS-mediated activation of the IKK branch of the Imd pathway. Expression of RHD prior to LPS treatment blocked efficient processing of Relish (Fig. 6B). Thus, Relish must inactivate at least one component that is shared by the JNK and IKK/Relish modules. Nevertheless, there is sustained expression of *attacin*, *dipteracin*, and other Relish target genes (Fig. 1A). The pattern of Relish target expression may reflect a stabilization of RHD in the nucleus. To test this idea, we prepared cytoplasmic and nuclear fractions of S2 cells at different times after LPS treatment and monitored the level of full-length and processed Relish in the two subcellular fractions. The processed form was found to translocate to the nucleus as early as 30 min after stimulation. Moreover, the amount of processed Relish in the nucleus remained above the basal level for at least 12 h, indicating that a transcriptionally active pool of processed Relish can be maintained in the absence of detectable cytoplasmic processing event (Fig. 6C).

Of the components shared by the JNK and IKK/Relish modules, we chose to examine TAK1, the MAPKKK that is common to both pathways. To assay TAK1 activity, we used a vector expressing Tak1 protein fused to the flu haemagglutinin (HA) epitope tag from the copper-inducible MT promoter and examined its ability to activate JNK in transiently transfected cells.

In control S2MK cells, copper-mediated induction of the HA-TAK1 construct resulted in gradually increasing levels of both Tak1 protein and activated JNK (Fig. 6D). In contrast, HA-TAK1 accumulated at only very low levels in S2RHD cells treated with copper, and there was a concomitant minor induction of JNK activation (Fig. 6D). To determine whether the lack of TAK1 accumulation in copper-treated S2RHD reflected increased degradation or decreased production, we carried out a pulse-chase experiment. As shown in Figure 6E, the turnover rate of TAK1, as determined after 8 h of copper treatment, was much higher in S2RHD cells than in S2MK cells. Taken together with our earlier findings, the shortened half-life of TAK1 and minimal JNK activation in cells expressing RHD provide strong support for the idea



**Figure 6.** Destabilization of Tak1 protein by Relish activation. (A) S2MK and S2RHD cells were treated with  $\text{CuSO}_4$  and then unstimulated or stimulated with 0.4 M NaCl for the length of time indicated. Phospho-JNK and FL Relish were analyzed by immunoblotting. (B) S2MK and S2RHD cells treated with  $\text{CuSO}_4$  were unstimulated or stimulated with LPS. FL Relish and its C-terminal domain (CTD) fragment generated by endoproteolytic processing were analyzed by immunoblotting. (C) S2 cells were treated with LPS for the length of time indicated. Cytoplasmic and nuclear protein fractions were prepared and analyzed by immunoblotting. (D) S2MK and S2RHD cells were transfected with HA-TAK1 and Flag-DJNK expression plasmids and then treated with  $\text{CuSO}_4$ . Protein lysates were prepared at the indicated time point and analyzed by immunoblotting. (E) S2MK and S2RHD cells were transfected with the HA-TAK1 expression plasmid and then treated with  $\text{CuSO}_4$  for 8 h. Cells were then pulse-labeled with [ $^{35}\text{S}$ ]methionine for 1 h and chased with nonradioactive medium for the time points indicated. HA-Tak1 protein was immunoprecipitated from protein lysates and analyzed by SDS-PAGE and autoradiography.

that Relish triggers negative feedback regulation of immediate early JNK signaling by directing the proteasome-mediated degradation of TAK1.

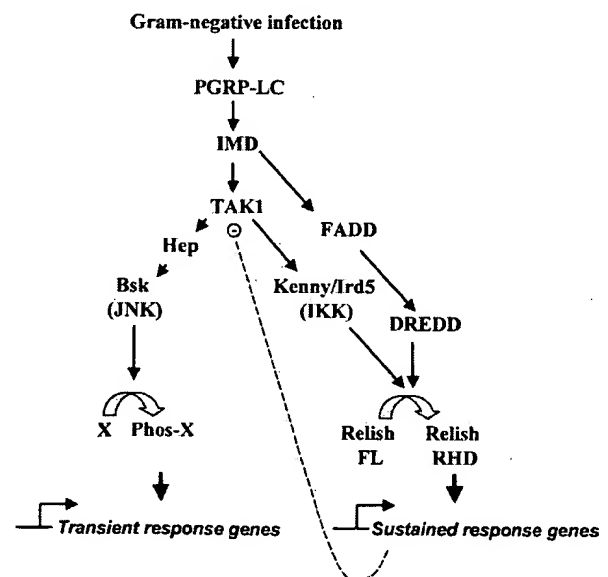
## Discussion

The JNK and IKK/Relish branches of the Imd pathway mediate distinct gene induction responses in *Drosophila* innate immunity (Fig. 7). After diverging downstream from TAK1, these two signaling cascades regulate two separate groups of target genes that are distinct in their induction kinetics and function. The IKK/Relish targets have been extensively characterized and most encode products whose role in innate immunity is relatively well established. On the other hand, the JNK-regulated, LPS-responsive genes represent a largely uncharacterized set of loci whose function in innate immunity is not clear. These genes exhibit transient induction kinetics, reaching a maximum ~1 h after induction. We found that the transient kinetics of the JNK target genes is controlled by the transient kinetics of the JNK module of the Imd pathway and that the IKK/Relish branch plays an active role in turning off JNK activity. Hence, the two seemingly independent branches of the Imd pathway are wired in such a way as to coordinate the temporal order of individual responses.

Our evidence indicates that Relish-mediated JNK inhibition involves proteasomal degradation of TAK1, the MAPKKK responsible for JNK activation in response to LPS. Treatment with proteasomal inhibitors or RNAi against a component of the proteasome complex results in sustained JNK activation during the LPS response. Furthermore, in cells expressing constitutively active Relish, the stability of TAK1 is greatly decreased. Based on these findings, we suggest that certain targets of Relish that are induced during immune responses facilitate destruction of TAK1 and switch off the JNK cascade (Fig.

7). The fact that cycloheximide and actinomycin D also block the down-regulation of JNK activity indicates the involvement of targets of Relish rather than Relish itself. The Relish target involved in this cross-talk likely increases the susceptibility of TAK1 to proteasomal degradation by direct targeting of TAK1 or by antagonizing factors responsible for TAK1 stabilization.

In considering this model, it should be noted that TAK1 is critically required for activating both IKK and JNK. Elimination of TAK1 during the LPS response thus turns off both of the downstream signaling cascades. Yet IKK/Relish target genes do not show a transient expression pattern, whereas JNK targets do. One possible explanation for this discrepancy lies in the nature of JNK



**Figure 7.** Model of branched Imd pathway.

and Relish activation. JNK is activated through its phosphorylation, a modification that is highly reversible. Thus, termination of the input that contributes to JNK phosphorylation is sufficient to result in its rapid inactivation, especially when one of the JNK targets encode a JNK phosphatase, Puckered. Relish, on the other hand, is activated through proteolysis, an irreversible modification. Once activated Relish enters the nucleus, it may remain bound to its target genes for some time even after termination of the upstream signal.

Interestingly, the antagonism between NF- $\kappa$ B and JNK signaling is evolutionarily conserved. In mice, inactivation of either IKK $\beta$  or NF- $\kappa$ B RelA (p65) in cells leads to a sustained JNK activation in response to TNF $\alpha$  (Karin and Lin 2002). The sustained JNK activation by TNF $\alpha$  has been associated with TNF $\alpha$ -induced apoptosis. Several independent studies have proposed different molecules as mediators for the NF- $\kappa$ B inhibition of JNK signaling in the TNF $\alpha$  pathway including XIAP (Tang et al. 2001), GADD45 $\beta$  (De Smaele et al. 2001), and reactive oxygen species (Sakon et al. 2003). Nevertheless, the underlying mechanism remains largely unknown (Liu 2003). As the *Drosophila* Imd pathway and the signaling pathway downstream of mammalian TNF receptor share many conserved features (Hoffmann and Reichhart 2002), a similar mechanism may govern the antagonistic relationship between IKK/NF- $\kappa$ B and JNK in both flies and mammals. Thus, the mechanistic insights gained from our studies in *Drosophila* should be of relevance to elucidating the mechanism that connects NF- $\kappa$ B to JNK signaling in mammals.

## Materials and methods

### S2 cell culture and treatment

Immunocompetent S2 cells were grown in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen). Cells were maintained as described previously (Sun et al. 2002). A commercial LPS preparation (Sigma; 055:B5) was dissolved in H<sub>2</sub>O and applied to cells at a final concentration of 10  $\mu$ g/mL. Chemical agents used in screening for an inhibitor of the RHD-mediated JNK inhibition were N-tosyl-L-phenylalanine, AEBSF, z-VAD-fmk, ac-YVAD-cmk, z-DEVD-fmk, z-FA-fmk, ac-LV-lysinal, diphenyleneiodonium chloride, bafilomycin A1, aminoguanidine, N<sup>G</sup>-monomethyl-L-arginine monoacetate, MG-132, and lactacystin (Calbiochem).

### Fly immune challenge

All *Drosophila* stocks were raised at room temperature on standard cornmeal, agar, and yeast medium. The *rel*<sup>E20</sup>, *rel*<sup>E38</sup>, *ird5*<sup>2</sup>, *imd*<sup>1</sup>, and *tak1*<sup>1</sup> mutations have been described previously (Hedengren et al. 1999; Georgel et al. 2001; Lu et al. 2001; Vidal et al. 2001). To study bacterial infection, adult flies were pricked with a fine glass needle dipped in a live culture of *E. cloacae* resuspended at high concentration in sterile Ringer's solution.

### Microarray analysis

S2 cells were grown in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum and 1% glutamine (Invit-

rogen). Ecdysone was added to a final concentration of 1  $\mu$ M before  $3 \times 10^6$  of these cells were plated in a 3-mL volume per well of a 6-well dish. Cells were induced with LPS 24 h later. At specified intervals, media was removed by aspiration and the cells harvested by scraping into 4°C PBS. Total RNA was isolated using the QIAGEN RNeasy kit and QIAshredder; final elution was done with water.

Affymetrix *Drosophila* gene chips were used for gene expression analysis. Briefly, double-stranded cDNA was synthesized from 5  $\mu$ g of total RNA. Biotin-labeled cRNA was synthesized using the MessageAmp aRNA kit (Ambion); 15  $\mu$ g of cRNA was fragmented and hybridized to each array. Each RNA sample was processed twice to obtain replicate biotin-labeled probes. Arrays were washed with the EukGW2 protocol on the GeneChip Fluidics Station (Affymetrix) and scanned using the Gene Array scanner (Affymetrix). Analysis of intensity data, including scaling, normalization, and treatment of replicates, were as described previously (De Gregorio et al. 2001).

### RNAi experiments

Templates for RNA transcription containing T7 promoter sequences flanking a cDNA fragment 200–1000 bp in length were generated by PCR. After in vitro transcription using the Megascript kit (Ambion), dsRNA was purified by ethanol precipitation and then dissolved in H<sub>2</sub>O. For RNAi, S2 cells were incubated with serum-free medium containing 25  $\mu$ g/mL dsRNA for 3 h. Full-length mouse interleukin-1 $\beta$  dsRNA, which is not homologous to any *Drosophila* gene, was used for the mock RNAi treatment. Serum was then added back to the culture, and cells were grown for an additional 72 h prior to protein and RNA analyses.

### Protein analysis

Whole-cell extracts for immunoblot analysis were prepared with lysis buffer (20 mM HEPES-KOH at pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 25 mM  $\beta$ -glycerophosphate, 2 mM EDTA, and protease inhibitors) and then subjected to SDS-PAGE. Proteins transferred to nitrocellulose membrane were probed with antibodies directed against the RHD and the C-terminal domain of Relish (from Dan Hultmark, Umeå University, Umeå, Sweden), anti-Cactus (from Ruth Steward, Rutgers University, Piscataway, NJ), anti-PolII (from John Lis, Cornell University, Ithaca, NY), and anti-phospho-JNK (Promega) antibodies, and the immunoreactive species were visualized with the ECL Western blot reagent (Pierce). The immune complex kinase assay was carried out as previously described (Sluss et al. 1996). For the pulse-chase experiment, 10<sup>7</sup> S2 cells were pulse-labeled for 1 h with 200  $\mu$ Ci of [<sup>35</sup>S]methionine in 0.5 mL of methionine-free M3 medium (Sigma) and then chased by adding 0.5 mL of M3 medium supplemented with 100 mM unlabeled methionine. HA-Tak1 protein was immunoprecipitated from the whole-cell lysates with 2  $\mu$ L of anti-HA antibody (Roche) and analyzed by SDS-PAGE and autoradiography.

### RNA analysis

Total RNA was isolated from S2 cells or whole flies using the RNAwiz reagent (Ambion). For RNase protection assays, 10  $\mu$ g of total RNA and 100,000 cpm of a <sup>32</sup>P-labeled antisense probe with a length of ~100 nt were hybridized, digested with a mix of RNases T1 and A, and recovered for gel electrophoresis using the RPA kit (Ambion). For real-time PCR analysis, cDNAs were synthesized with the Superscript II reverse transcriptase system (Invitrogen). An amount of cDNA equivalent to 0.2  $\mu$ g of total

RNA was subjected to 40 cycles of PCR amplification consisting of a 15-sec incubation at 95°C and a 1-min incubation at 60°C. Output was monitored using SYBR Green core reagents and the ABI Prism 7700 System (PE Applied Biosystems). All the results were normalized to the *rp49* mRNA level. Individual primer sequences are available upon request.

## Acknowledgments

We thank Dan Hultmark, John Lis, and Ruth Steward for providing antisera; Deborah Kimbrell, Dan Hultmark, and Bruno Lemaitre for the mutant fly lines; Tony Ip, Neal Silverman, Tom Maniatis, and Jun R. Huh for providing plasmids; Andrea D'Auria for technical assistance; and Paul Spellman for assistance and advice on microarray analysis. This work was supported by a BIOSTAR grant (00-10120) and an NIH grant GM50545 to S.A.W. and by NIH grants CA76188 and AI43477 to M.K. J.M.P. is supported by a postdoctoral fellowship from the Human Frontier Science Program. M.K. is an American Cancer Society Research Professor.

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## *P* element insertion-dependent gene activation in the *Drosophila* eye

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Contributed by Gerald M. Rubin, February 19, 1997

**ABSTRACT** Insights into the function of a gene can be gained in multiple ways, including loss-of-function phenotype, sequence similarity, expression pattern, and by the consequences of its misexpression. Analysis of the phenotypes produced by expression of a gene at an abnormal time, place, or level may provide clues to a gene's function when other approaches are not illuminating. Here we report that an eye-specific, enhancer–promoter present in the *P* element expression vector pGMR is able to drive high level expression in the eye of genes near the site of *P* element insertion. Cell fate determination, differentiation, proliferation, and death are essential for normal eye development. Thus the ability to carry out eye-specific misexpression of a significant fraction of genes in the genome, given the dispensability of the eye for viability and fertility of the adult, should provide a powerful approach for identifying regulators of these processes. To test this idea we carried out two overexpression screens for genes that function to regulate cell death. We screened for insertion-dependent dominant phenotypes in a wild-type background, and for dominant modifiers of a *reaper* overexpression-induced small eye phenotype. Multiple chromosomal loci were identified, including an insertion 5' to *hid*, a potent inducer of apoptosis, and insertions 5' to *DIAP1*, a cell death suppressor. To facilitate the cloning of genes near the *P* element insertion new misexpression vectors were created. A screen with one of these vectors identified *eagle* as a suppressor of a rough eye phenotype associated with overexpression of an activated *Ras1* gene.

Mutational inactivation is a powerful tool for understanding the role of a gene product in a specific process. However, this approach is limited by the fact that the majority of genes do not have an easily assayable loss-of-function phenotype; that is, mutations in most genes are phenotypically silent under laboratory conditions. Second, any observed phenotype only reflects that part of a gene's function that is not compensated for by other genes and pathways. Finally, many genes are required for multiple aspects of normal development or adult function. These limitations make it difficult to address the function of a gene late in development, or in the adult if the gene is also required at an earlier stage for cell proliferation, cell survival, or differentiation (reviewed in ref. 1).

An alternative approach to understanding gene function is to characterize phenotypes resulting from tissue-specific expression of individual genes in tissues where they are not normally expressed or are expressed at elevated levels at a normal site of expression. Such misexpression may create phenotypes, whereas mutational inactivation does not. Misexpression also provides a way of asking if a gene product is able to direct a particular process or alter the output of a signaling

pathway in a particular tissue. Genes identified in one tissue as signal modifiers by overexpression phenotypes are likely to be important regulators, even if the gene is normally not expressed in that tissue. These genes might be useful in gene therapy, where a major goal is to identify genes that can modify signaling pathways in novel contexts. Misexpression of individual, cloned genes is a valuable approach for identifying developmental regulators or signaling molecules, but it requires that one have the full-length candidate gene in hand and that these genes be introduced into the germ line one at a time. Also, selection of appropriate candidate genes requires prior knowledge about what genes are likely to be important for the process under study. In contrast, misexpression of random genes allows one to search for genes that can affect a process without preconceptions.

Important developmental regulators and oncogenes have been identified as a result of fortuitous tissue-specific gene overexpression due to chromosomal aberrations or insertions of retrotransposons or transposable elements that bring genes under the control of novel transcriptional regulators (2–5). Screens designed to identify genes based on misexpression-dependent phenotypes can be carried out in several ways. In transfectable single-cell organisms such as yeast, or in mammalian cell culture, overexpression of random clones from cDNA libraries can be used to identify genes that can alter cell fate or modify the output of specific signaling pathways (6–8). In intact plants and animals, tissue-specific overexpression of unknown genes can be brought about by using insertions of transposable elements containing tissue-specific enhancers or enhancer–promoters to drive the expression of nearby genes. The molecular markers provided by the insertions facilitate cloning of the expressed genes. This “activation tagging” approach has also led to the identification of developmental regulators and oncogenes (9–11). *Drosophila* is an ideal system in which to carry out activation tagging screens because transposable elements (*P* elements) can be mobilized throughout the genome at a high frequency, in a controlled fashion (12), and because mutagenic *P* elements have a preference for insertion near the 5' end of a gene (13).

Previously we described a *P* element expression vector pGMR that drives eye-specific expression of cloned genes (14). Here we show that sequences within pGMR are also able to drive the eye-specific expression of endogenous genes near the site of *P* element insertion. We generated 500 new insertions of the empty GMR vector. Four percent of these insertions are associated with dominant eye phenotypes, and another 1.6% act as modifiers of an excess eye cell death phenotype resulting from expression of the apoptosis inducers *reaper* (*rpr*) or *hid*. We have created a new *P* element vector, GMREP, that facilitates cloning of genes identified through eye misexpression phenotypes.

### MATERIALS AND METHODS

**Vector Construction.** pGMREP (Fig. 1) was created by ligating an *XhoI*–*PstI* fragment of pGMR that contains the

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Abbreviation: HA, hemagglutinin.

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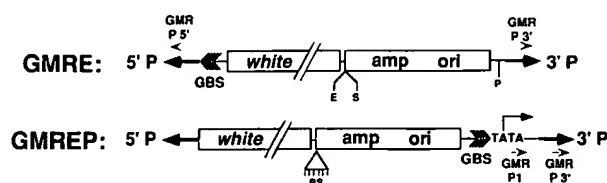


FIG. 1. Maps of the vectors GMRE and GMREP. The positions of the *white* marker gene (*white*), sequences for plasmid rescue (*amp* and *ori*), and the pentamer of GLASS binding sites (GBS) are indicated. Restriction endonuclease sites for plasmid rescue are represented by short vertical lines. Primer sequences used for PCR are indicated by small arrowheads (GMR P1 and GMR P3'). In GMREP, the TATA box and 5' splice donor sequences are indicated. The Bluescript polylinker (BS) is indicated as a triangle with vertical lines.

binding sites for the transcription factor GLASS and the *hsp70* TATA box into *XhoI*-*PstI* cut Bluescript (Stratagene). Sites downstream of the GLASS binding sites present in the pGMR fragment were removed by cutting with *SalI* and *BamHI*, followed by blunting with T4 DNA polymerase and ligation to create GMR-Bluescript. To create a 5' splice donor site downstream of the *hsp70* TATA box, a fragment of *glass* genomic DNA surrounding the first exon 5' splice donor site (nucleotides 4301–4481; ref. 15) was amplified using primers 5'-CGCTGCAGCTACTTAAACCGAGTCTTCG and 5'-GGAGATCTTTCTTTCTTCTTTTATTGCAGATTT. The product was cut with *BglII*, treated with T4 DNA polymerase, and then cut with *PstI*. The product was then ligated into GMR-Bluescript that had been cut with *XbaI*, treated with T4 DNA polymerase, and then cut with *PstI*. A *KpnI*-*NotI* fragment containing the glass binding sites, TATA box, and 5' splice donor site was shuttled into the vector pUAST (16) and removed as an *XbaI*-*BglII* fragment, which was cloned into the *P* element transformation vector PEG117 (17) that was cut with *XbaI* and *BamHI*. Restriction endonuclease sites 5' to the enhancer-promoter were removed by cutting with *XbaI* and *KpnI*, treating with T4 DNA polymerase and then religation. The *NotI* site 3' to the enhancer-promoter complex was removed by cutting with *NotI*, blunting with T4 DNA polymerase, and religation. To facilitate plasmid rescue of genomic DNA flanking the 3' *P* element end, a fragment containing the Bluescript polylinker was amplified by PCR using the Bluescript universal and reverse primers, blunted with T4 DNA polymerase, and cloned into PEG117 containing the enhancer-promoter construct cut with *SacII* and blunted with T4 DNA polymerase. All sites in the polylinker except *PstI* and *HindIII* are usable for plasmid rescue of genomic DNA flanking the 3' *P* element end. The polylinker is oriented such that sequencing with the T3 primer will read into genomic sequence following plasmid rescue.

pGMRE (Fig. 1) was created by cloning an *XhoI*-*SalI* fragment of pGMR that contained the pentamer of the GLASS binding sites into *XhoI*-*SalI*-cut Bluescript in a way that the *XhoI* and *SalI* sites are retained. A *NotI*-*KpnI* fragment of Bluescript that contains the GLASS binding sites was isolated and ligated into *NotI*-*KpnI* cut PEG117. *EcoRI* and *SacII* can be used for plasmid rescue of genomic DNA flanking the 3' *P* element end. *PstI* can be used to rescue genomic DNA flanking the 5' *P* element end.

The intracellular domain of the human FAS transmembrane receptor is able to transduce a cell death signal on multimerization (18). To create a potentially constitutively active FAS receptor we fused a sequence coding for the extracellular domain of the influenza hemagglutinin (HA), which forms trimers (19), to the transmembrane and intracellular domain of human FAS, thereby generating a construct known as HAFAS, which we hoped would transduce a ligand independent FAS death signal (details provided on request).

**Transformation and Screening.** Flies transgenic for pGMR, pGMRE, and pGMREP were generated using standard procedures (20), and insertions of each of these elements on the X chromosome were identified. Autosomal insertions were generated by crossing females with insertions on the X chromosome with males carrying a stable source of *P* element transposase activity (21). Progeny were then outcrossed to *w<sup>1118</sup>*. Insertions on the autosomes were identified as red-eyed males in the subsequent generation. Males and females with dominant eye phenotypes were balanced for the appropriate chromosome. Most autosomal insertions did not give rise to dominant phenotypes at the level of the dissecting microscope. These insertions were kept outcrossed to *w<sup>1118</sup>*. Individual transformant males (heterozygotes for the insertion) were crossed to flies that were either *GMR-rprM*/TM6B, *GMR-rprS*/TM6B (22), or *sev-Ras1<sup>V12</sup>* inserted on a CyO chromosome (CR2/*Adv*; see ref. 23). The progeny were scored for the presence of flies whose eye phenotype differed from that of *GMR-rprM*, *GMR-rprS*, or CR2 alone. Modifiers were balanced and characterized as described in the text. *th4* and *th5* were used to test the 72D insertions GMR228 and GMR355 for allelism (22). *eg<sup>1</sup>* and *eg<sup>2</sup>* were used to test 79A3-4 GMRE28 excision lines for allelism (24).

**RNA and DNA Isolation and Characterization.** To isolate DNA surrounding the site of pGMR insertion, inverse PCR was performed. Genomic DNA from *P* element lines was cut with *Sau3A*, diluted, and circularized with T4 DNA ligase. Primers 5'-GCATGTCCTGGGGTTTGAAT (Pry4) and 5'-CTTGCCGACGGGACCACCTTATGTTATT (GMR P3') extend in opposite directions within a *Sau3A* fragment that contains the 3' *P* element end. PCR was carried out with these primers; products were blunted with T4 DNA polymerase and cloned into *SmaI* cut Bluescript. They were sequenced by the chain termination method (25) using the Automated Laser Fluorescence system (Pharmacia).

To determine the location of the GMRE28 *P* element with respect to the *eagle* gene, plasmid rescue of flanking genomic DNA was carried out using *SacII*. By probing DNA blots of *EcoRI* digests of cosmids 8 and 27 (see ref. 24) with this plasmid rescue fragment we were able to place the *P* element 400–500 bases 5' to the *eagle* transcription unit, with the 3' *P* element end closest to *eagle* (see Fig. 5G).

PCR assays were performed to determine if chimeric transcripts extending from within the *P* element into the surrounding genomic region were being generated. RNA was isolated from 50 eye-antennal imaginal discs using the Micro-Scale Total RNA separator kit (Clontech, Palo Alto, CA). cDNA was generated from this RNA using the Clontech Marathon cDNA amplification kit. Gene-specific primers were used to prime cDNA synthesis: 5'-AATATATTGTTCTTGTGTC-CCGTC (hid P2, see Fig. 3D); 5'-TTGAATTTGAGGACT-TGGGTGCGC (DIAP P2, see Fig. 4G); and 5'-GCAGCCTTCACATGTAAATGCC (*eagle* P2, see Fig. 5G). PCR was then carried out using this cDNA as a template. One primer of each primer pair was a gene-specific primer located 3' to a large intron in the gene of interest, extending toward the 5' end of the gene: 5'-AACCGTCACAACAGTTGGCCAAGT-GAA (hid P1, see Fig. 3C); 5'-TGGCGAGGCCACCATGACCGC (DIAP P1, see Fig. 4G); and 5'-GCACACTTTG-CACAGCTGGTTCAT (*eagle* P1, see Fig. 5G). The second primer was either 5'-CGTCGCTAAGCGAAAGCTAAG-CAA (GMR P1), present just 3' to the *hsp70* transcription start site, or 5'-CTTGCCGACGGGACCACCTTATGTTATT (GMR P3'), present just 5' to the 3' *P* element end (see Fig. 3C).

**Histology.** Scanning electron microscopy (26), fixation and sectioning of adult eyes (27), and tissue *in situ* hybridizations (28) were performed as described previously.

## RESULTS AND DISCUSSION

We are interested in identifying and characterizing cell death signal transduction pathways in the fly. One approach we have taken is to express, specifically in a nonessential tissue, such as the eye, molecules that might be expected to alter the normal pattern of cell death. If expression of these molecules alters normal cell death signaling we can use the resulting phenotypes present in the adult as backgrounds in which to carry out genetic screens for *Drosophila* cell death regulators. To carry out this approach we made the pGMR expression vector, which contains a pentamer of binding sites for the GLASS transcription factor derived from the Rh1 promoter and TATA box sequences from the *Drosophila hsp70* promoter, cloned into the CaSpeR-hs vector (14). Sequences placed downstream of these sites are transcribed in a similar pattern to *glass* expression, in and posterior to the morphogenetic furrow during larval and pupal eye development (29). Pattern formation in the eye occurs during this same period as a series of inductive events in and posterior to the morphogenetic furrow during which cells must choose to differentiate, proliferate, or die (30).

As a part of our efforts to activate cell death signaling in the fly, we generated a large number of GMR transformants (about 100) expressing a transcript coding for a chimeric protein called HAFAS (GMR-HAFAS; described in *Materials and Methods*) that we hoped would transduce a cell death signal. Most lines transgenic for this construct have no visible adult eye phenotype. However, in one line (GMR-HAFAS110) the flies have very small eyes (Fig. 2B). This phenotype is suppressed by decreasing the dose of *glass* (Fig. 2C), suggesting that it is the result of *glass*-dependent transcription. The phenotype is completely suppressed, and eye size restored to normal, by coexpression of the baculovirus cell death inhibitor, p35 (Fig. 2D). P35 blocks cell death in multiple

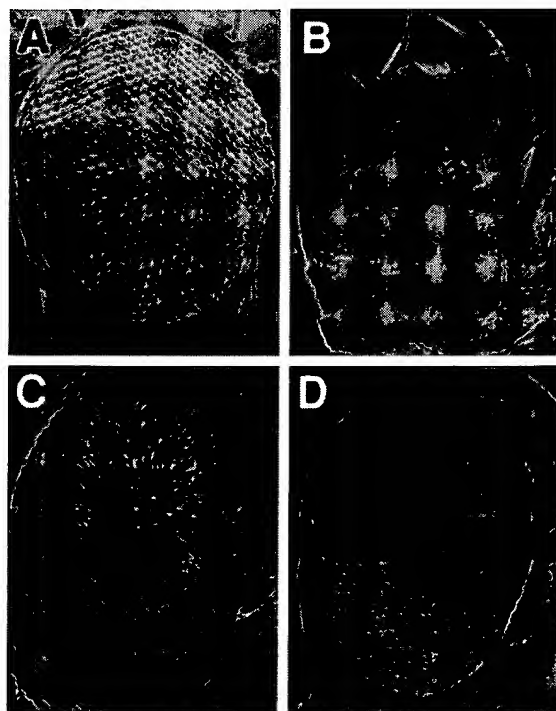


FIG. 2. Scanning electron micrographs of adult eyes of the following genotypes are shown: wild-type (A); GMR-HAFAS110/+ (B); GMR-HAFAS110/*gl60j* (C); GMR-HAFAS110/GMR-p35 (D). Flies with the GMR-HAFAS110 insertion (B) have small eyes due to excess cell death in the developing eye. This phenotype is partially suppressed by removing one copy of *gl* (C) and completely suppressed by coexpressing baculovirus p35 (D).

contexts in *Drosophila*, including death due to overexpression of the cell death activators *rpr*, *hid*, and *grim* (22, 31–33). These results indicate that *glass*-dependent activation of a cell death signaling pathway is occurring in the GMR-HAFAS110 line. Because most insertions of this construct have no visible phenotype in the adult, it is unlikely that cell death activation is due to expression of the HAFAS chimera. The GMR-HAFAS110 *P* element is located in the 75C1-2 cytological region. This region contains the *rpr*, *hid*, and *grim* genes, each of which is capable of inducing a cell death-dependent small eye phenotype when overexpressed (22, 31–34). The GMR-HAFAS110 *P* line is semilethal in trans to a chromosomal deletion for the 75C region, suggesting that it has inserted near an essential gene. Sequencing of genomic DNA surrounding the insertion site shows that it is inserted 131 bases 5' to the longest *hid* cDNA described (ref. 31; Fig. 3C). Tissue *in situ* hybridizations to third instar eye imaginal discs with a *hid* cDNA probe show that in wild-type eye imaginal discs *hid* is

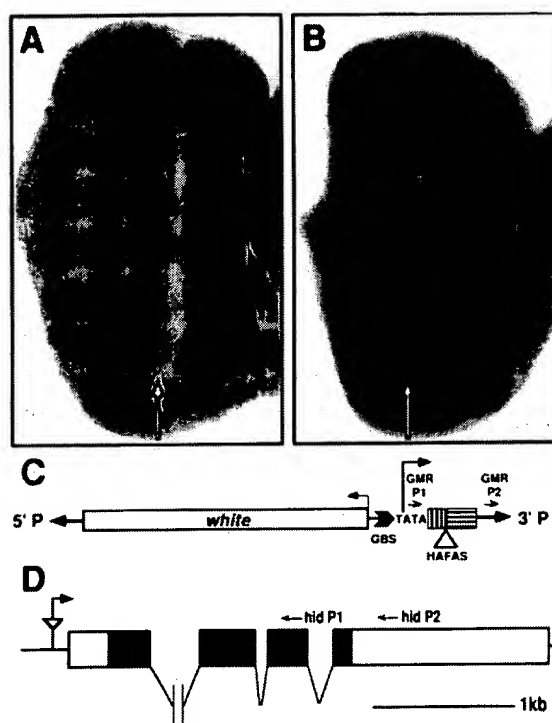


FIG. 3. Misexpression of the *hid* gene by the GMR-HAFAS110 insertion. The *hid* expression pattern in eye-antennal discs from wild type (A) and GMR-HAFAS110/GMR-HAFAS110 (B) larvae are shown. The *hid* transcript is expressed uniformly in the wild-type eye imaginal disc (A) and at high levels in and posterior (left) to the morphogenetic furrow (arrow) in GMR-HAFAS110 eye imaginal discs (B). (C) Map of the GMR-HAFAS construct. The expression vector pGMR contains the white marker gene (*white*), a multimer of glass binding sites (GBS), TATA sequences from the *hsp70* promoter (TATA), approximately 200 bases of 5' untranslated region (box marked with vertical bars), a polylinker into which the HAFAS construct was cloned (HAFAS is not drawn to scale), and the *hsp70* 3' untranslated region (box marked with horizontal bars). The direction of transcription from the *hsp70* promoter is indicated. The *P* element ends are indicated as boldface arrows. The location of the *P* element primers used to detect chimeric transcripts are indicated with small arrowheads (GMR P1 and GMR P2). (D) Map of the genomic region at the site of insertion of the GMR-HAFAS110 *P* element. GMR-HAFAS110 is inserted 131 base pairs 5' to the longest *hid* cDNA (described in ref. 26). The *P* element is indicated by the triangle and is oriented such that transcription from the *hsp70* TATA box reads through the *P* element 3' end and into the 5' end of the *hid* transcription unit as indicated by the arrow. The locations of primers used for cDNA synthesis (*hid* P2) and PCR (*hid* P1) are indicated on the map. The introns are not drawn to scale.

expressed at uniform low levels (Fig. 3A) but at much higher levels in and posterior to the morphogenetic furrow in eye imaginal discs from GMR-HAFAS110 flies (Fig. 3B). These observations suggest that the multimerized GLASS binding sites present in the GMR-HAFAS110 line are acting to drive eye-specific expression of the endogenous *hid* gene. By carrying out PCR on cDNA generated from GMR-HAFAS110 eye-antennal disc total RNA using *P*-element- and gene-specific primers (see *Materials and Methods* and Fig. 3C), we were able to detect chimeric transcripts containing sequences from the *P* element 3' end and the *hid* coding region. We were unable to detect chimeric transcripts initiating at the transcription start site downstream from the *hsp70* TATA box, but we were able to detect chimeric transcripts that contained *P* element sequences closer to the 3' *P* end. These chimeric transcripts may initiate at the *hsp70* promoter, but be unstable, perhaps due to the presence of sequences from the *hsp70* 3' untranslated region; alternatively, chimeric *hid* transcripts may be generated using an cryptic promoter closer to the 3' *P* element end.

Because mutagenic *P* element insertions tend to occur in or near the 5' end of genes (13), our observations with the HAFAS110 line suggest that it should be possible to sample a significant fraction of the genome for genes that can affect some aspect of eye development when overexpressed by mobilizing pGMR throughout the genome.

To test this idea we carried out several screens in which new insertions of empty pGMR were created. In one screen we looked for dominant phenotypes as a result of mobilization of pGMR to new sites on the autosomes. In a second screen these autosomal insertions were then scored for their ability to act as dominant modifiers of *rpr* overexpression-induced small eye phenotypes (GMR-*rprM* and GMR-*rprS*) used previously in a screen for genes in which reduction in function modified the extent of cell death (22). A dominant eye phenotype was observed in 4% of the lines tested (19 of 500), ranging from a very small eye to various degrees of roughness (data not shown). These phenotypes are suppressed by removing one copy of *glass*, therefore indicating that they are due to *glass*-dependent expression, presumably of nearby genes.

In crosses to GMR-*rprM* or GMR-*rprS* flies five enhancers and two suppressors were identified (Fig. 4 A–C). The two suppressor lines (GMR228 and GMR355) each contain a single *P* element that maps to the 72D1-2 cytological region. Complementation crosses identify GMR228 as a lethal allele of *thread* (*th*), which codes for the DIAP1 protein, a dose-dependent suppressor of *rpr*- and *hid*-dependent cell death (22). In contrast to the GMR228 *th* allele, other lethal alleles of *th* act as enhancers of *rpr* and *hid*-dependent cell death, due to a decrease in DIAP1 activity. The GMR355 line is semilethal when homozygous but complements lethal *th* alleles for viability. The GMR228 *P* element is inserted at base 34 of the 5' untranslated region of the largest DIAP1 cDNA isolated (22), whereas the GMR355 *P* element is inserted 70 bases 5' to this cDNA. (Fig. 4G). Tissue *in situ* hybridizations with a DIAP1 probe show that DIAP1 mRNA is expressed at uniform low levels in wild-type third instar eye-antennal discs (Fig. 4D) but at much higher levels in and posterior to the morphogenetic furrow in discs from GMR228 (Fig. 4E) and GMR355 flies (Fig. 4F). In both lines the *P* element is inserted such that transcription from the GMR *hsp70* TATA box would extend into the 5' end of the DIAP1 gene (Fig. 4G). PCR using *P* element and DIAP1 specific primers (see *Materials and Methods*) show that chimeric transcripts are being created (data not shown). Therefore, the cell death suppression seen in these lines is due to the GMR insertion-dependent expression of chimeric DIAP1 transcripts.

The above results show that insertion of pGMR results in a high frequency of eye phenotypes. Characterization of the above three lines suggests that pGMR is acting as an enhancer-

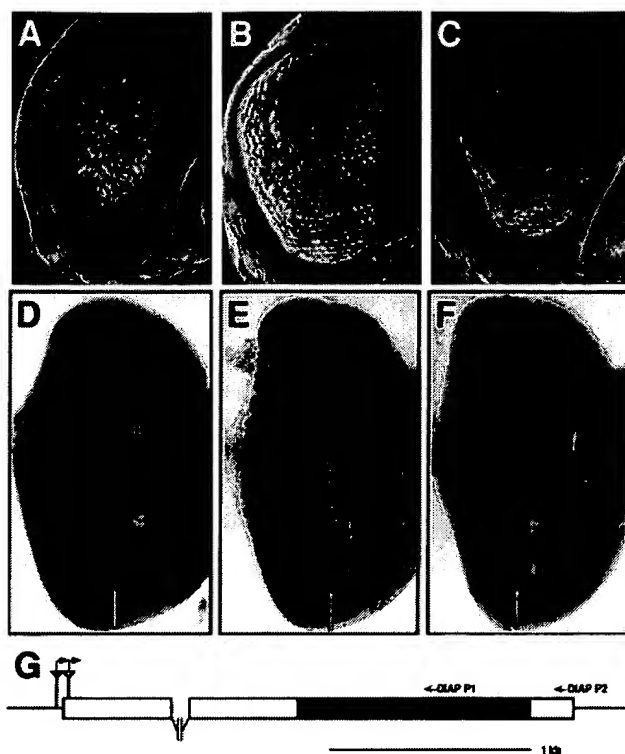


FIG. 4. GMR insertions near the DIAP1 gene act as strong suppressors of a GMR-*rpr*-dependent small eye phenotype and express high levels of the DIAP1 gene in the developing eye. The following genotypes are shown: GMR-*rprS*/TM6B (A); GMR-*rprS*/GMR228 (B); GMR-*rprS*/GMR355 (C); wild type (D); GMR228/TM6B (E); GMR355/TM6B (F). GMR-*rpr* expression results in a cell death-dependent small eye phenotype (A), which is suppressed in the presence of the GMR228 (B) and GMR355 (C) chromosomes. DIAP1 transcript levels are uniform throughout the wild-type eye-imaginal disc (D) but are elevated posterior to the morphogenetic furrow in the GMR228 (E) and GMR355 (F) lines. (G) Diagram of the DIAP1 chromosomal region. The *P* element in the GMR228 line is inserted after base 34 in the DIAP1 5' untranslated region and 70 bases upstream of the DIAP1 5' untranslated region in the GMR228 line. Both *P* elements (triangles) are oriented with the 3' *P* end nearest the DIAP1 gene. The direction of *hsp70* transcription is indicated by the raised, rightward pointing arrows. Small arrows above the map indicate the primers used to prime cDNA synthesis (DIAP P2) and to carry out PCR (DIAP P1) to detect the presence of chimeric DIAP1 transcripts.

promoter vector; eye-specific transcription is driven through the 3' *P* element end and into the surrounding genomic region. pGMR is not ideal for overexpression screens because it contains extraneous sequences (the *hsp70* 3' untranslated region) between the *hsp70* TATA box and the 3' *P* element end and because it lacks plasmid rescue capability. To facilitate further screens of this kind, and to determine if promoter sequences are required to generate a high frequency of GLASS multimer-dependent eye phenotypes, we constructed and tested two new vectors. In one vector, GMREP, the GLASS binding site pentamer, *hsp70* TATA box sequences, and a 5' splice donor sequence are located near the 3' *P* element end (Fig. 1). In a second vector, pGMRE, the GLASS binding site multimer alone is present, located near the *P* element 5' end (Fig. 1). Both vectors have plasmid rescue sequences and multiple unique restriction endonuclease sites to facilitate the cloning of nearby overexpressed genes.

Mobilization of pGMREP to the autosomes resulted in *glass*-dependent, dominant phenotypes with a frequency of 8–10%, more than twice that seen with pGMR. This higher frequency may be due to some combination of increased translatability of GMREP transcripts, which have a much shorter 5' untranslated region, increased transcript stability

due to the lack of sequences from the *hsp70* 3' untranslated region, or splicing of transcripts originating from GMREP intron insertions to downstream coding exons. Mobilization of pGMRE resulted in a very low frequency (1/1000) of dominant eye phenotypes. GMRE lines were also scored for their ability to modify a GMR-*rpr* induced small eye phenotype or a rough eye phenotype associated with expression of an activated *Drosophila Ras1* gene under control of the *sevenless* enhancer-promoter (*sev-Ras1<sup>V12</sup>*; see ref. 35). Several enhancers of GMR-*rprS* and one suppressor of *sev-Ras1<sup>V12</sup>* (GMRE28) were identified. The *sev-Ras1<sup>V12</sup>* suppressor was characterized further.

*sev-Ras1<sup>V12</sup>* flies have a rough eye phenotype (Fig. 5A) associated with extra R7 photoreceptors and ommatidial fusions (35). GMRE28 dominantly suppresses this rough eye phenotype (Fig. 5B), and there appear to be fewer ommatidial fusions (data not shown). The GMRE28 suppressor phenotype is *P* element-associated, because excision of the *P* element results in a loss of the suppressor phenotype (data not shown); this phenotype is also *glass*-dependent and dosage-sensitive. Cytologically, the GMRE28 *P* element maps to 79A3-4. Excision lines were generated with the intention of isolating loss-of-function mutations in the ectopically expressed gene at 79A3-4. A high percentage of these lines exhibited a held-out wing phenotype similar to that associated with mutations-

affecting the *eagle* gene (24). The GMRE28 *P* element is inserted approximately 400–500 bases 5' to the *eagle* transcription unit (Fig. 5G) and complements *eagle* alleles. The GMRE28-dependent *sev-Ras1<sup>V12</sup>* suppression is not due to a decrease in *eagle* function because chromosomal deletions for the region and loss of function *eagle* alleles do not suppress the *sev-Ras1<sup>V12</sup>* phenotype (data not shown).

To determine if the suppression is due to *eagle* overexpression in the eye we first carried out tissue *in situ* hybridizations using an *eagle* probe. In wild-type eye imaginal discs, *eagle* RNA is present at undetectable levels (Fig. 5D). In GMRE28 eye discs *eagle* is easily detected in the morphogenetic furrow and is just detectable posterior to the furrow (Fig. 5E). We then fused a full length *eagle* cDNA directly to the GMR enhancer-promoter and introduced this construct (GMR-*eagle*) into the germline. GMR-*eagle* transformants have higher levels of *eagle* expression in the eye imaginal disc posterior to the morphogenetic furrow (Fig. 5F), and there is a correspondingly higher degree of suppression of the *sev-Ras1<sup>V12</sup>* rough eye phenotype (Fig. 5C). Thus *eagle* overexpression acts as a *sev-Ras1<sup>V12</sup>* suppressor. The GMRE vector was designed to lack an outwardly pointing promoter and, as expected, RT-PCR experiments (see *Materials and Methods*) failed to detect hybrid *P* element-*eagle* transcripts.

Mutations that alter the *sev-Ras1<sup>V12</sup>* rough eye phenotype include alterations in genes involved in *Ras1* pathway signaling, *Ras1* posttranslational modification, and *sevenless*-dependent transcription (23). Our data do not allow us to determine which of these processes is affected by *eagle* misexpression. Loss-of-function *eagle* phenotypes in the embryo suggest a requirement for the proper differentiation of a small number of cells in the CNS (24). By tissue *in situ* hybridization, *eagle* does not appear to be differentially or transcriptionally activated in photoreceptors or other cells of the eye disc (Fig. 5D). However, loss of function *eagle* phenotypes have not been characterized in the developing eye, therefore it is not known if *eagle* plays a role in normal eye development. *eagle* encodes a zinc finger protein sharing homology with steroid receptor family members, suggesting that it may function to regulate transcription, but its targets are unknown (24).

**Concluding Remarks.** We have shown that an eye-specific enhancer-promoter complex, when mobilized throughout the genome in a *P* element, results in a high frequency of misexpression-dependent phenotypes. In the case of the GMR and GMREP vectors, these phenotypes result from the production of chimeric transcripts, initiating within the *P* element and extending into the surrounding genomic region. The utility of this approach was demonstrated in screens for genes important in cell death signaling. Because the phenotypes generated by *P* element insertion-dependent gene activation are due to the production of chimeric transcripts, in most cases the *P* element will be near the gene being expressed. Loss-of-function mutations in these genes can be made by imprecise excision of the *P* element. In cases such as the GMR228 insertion into *DIAP1*, where the normal expression of the gene has been inactivated, the loss-of-function phenotype can be scored directly in tissues other than the eye, where the GMR enhancer-promoter is inactive.

A related approach for large scale random gene misexpression in *Drosophila* has been described by Rorth (36). In this method, Gal4 expressed in specific patterns is used to drive the expression of genes near the insertion site of a *P* element containing Gal4 binding sites and a promoter sequence near one *P* element end. This system is very versatile because it allows one to test gene activating insertions for misexpression-dependent phenotypes in multiple tissues by crossing the Gal4 binding site insertion lines to flies in which Gal4 is expressed in different spatial and temporal patterns. High level Gal4 expression in the eye can disrupt normal development (37), but these effects can be mitigated by using lines that express lower

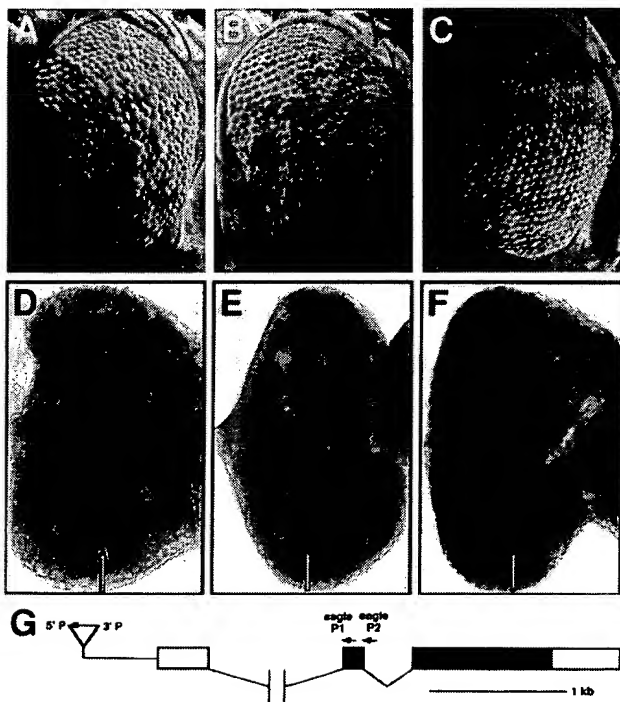


FIG. 5. A GMRE insertion near the *eagle* gene acts as a suppressor of the *sev-Ras1<sup>V12</sup>*-dependent rough eye phenotype by directing *eagle* expression in the developing eye. The following genotypes are shown: *sev-Ras1<sup>V12</sup>* (CR2)/+ (A); *sev-Ras1<sup>V12</sup>* (CR2)/GMRE28 (B); *sev-Ras1<sup>V12</sup>* (CR2)/GMR-*eagle* (C); wild type (D); GMRE28/+ (E); GMR-*eagle*/+ (F). Expression of *sev-Ras1<sup>V12</sup>* (CR2) results in a rough eye phenotype (A). This phenotype is mildly suppressed in the presence of the GMRE28 chromosome (B) and more strongly suppressed by GMR-*eagle* expression (C). The degree of *sev-Ras1<sup>V12</sup>* rough eye suppression is correlated with the level of *eagle* expression: wild-type eye imaginal discs express little if any *eagle* (D) discs from GMRE28 flies express *eagle* at higher levels in the morphogenetic furrow (arrow), and to some extent posterior to the morphogenetic furrow (E); and discs from GMR-*eagle* flies express high levels of *eagle* in and posterior to the morphogenetic furrow (F). (G) Map of the *eagle* genomic region. GMRE28 is inserted approximately 500 bases 5' to the *eagle* transcription unit as indicated. Noncoding cDNA sequences are indicated by open boxes and coding sequences by filled boxes.



levels of Gal4. Loss of function phenotypes can be characterized in the absence of Gal4 expression. In contrast, when using GMREP, one is primarily limited to screening for phenotypes in the developing eye. However, because insertions can be scored for eye phenotypes directly on generation, screens can be carried out somewhat more quickly. Overexpression-dependent phenotypes in other tissues or at other times can be examined by crossing GMREP lines to flies expressing GLASS under heat shock control (14, 29). The higher frequency of phenotypes seen with GMREP insertions (8–10%) compared with insertions of a Gal4 enhancer–promoter vector in which transcription is driven by sevenless-Gal4 (4%; ref. 36) may reflect the fact that GLASS is expressed earlier than *Sevenless* during eye development and in more cell types.

Misexpression screens with either system will be useful for identifying developmental regulators or other signaling molecules in which activity is transcriptionally regulated by subtly altering the levels or timing of their expression in tissues in which they are normally expressed. Misexpression screens can also identify genes that can function to regulate development or other cellular functions in a specific tissue, even if the gene is normally not expressed in that tissue. A limitation of the misexpression approach is that many genes can disrupt normal development when misexpressed at high levels. Therefore, a critical factor governing the successful implementation of this approach will be the ability to carry out efficient secondary analyses to identify genes that are directly affecting a process of interest. One way to target a particular pathway is to screen for suppressors of an existing phenotype that has been generated by activation of that pathway, as we have demonstrated here for the *GMR-rpr* and *sev-Ras<sup>VT2</sup>* phenotypes. Moreover, by screening for restoration of a more normal eye phenotype, the background of nonspecific effects should be greatly reduced.

We thank Todd Lavery for chromosome *in situ* hybridizations, Paula Sicurello for scanning electron microscopy, Chris Suh for sequencing, Kristen White (Massachusetts General Hospital) for providing some initial mapping data that placed the GMR-HAFAS110 insertion near *hid*; Henry Chang for preparing the figures; and Alan C. Spradling, Pernille Rorth, and Ilaria Rebay for helpful comments on the manuscript. This work was supported by the Howard Hughes Medical Institute (G.M.R.), and by an American Cancer Society, California Division, Senior Postdoctoral Fellowship (B.A.H.).

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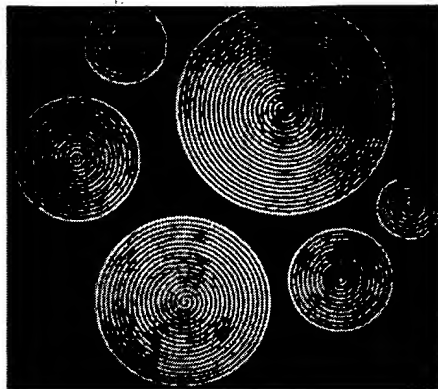


Figure 1 Pattern formation in Rayleigh–Bénard convection. Shadowgraphs of a horizontal fluid layer heated from below reveal large spiral patterns (dark corresponds to warm, rising fluid and light to cold, sinking fluid)<sup>7</sup>. Despite the underlying non-equilibrium nature of this process the spirals are surprisingly stable and regular.

structures seen in Rayleigh–Bénard convection of a fluid heated from below (Fig. 1).

Such striking results are emerging from

the increasing activity in studies of non-equilibrium systems<sup>2–6</sup>. Our fundamental understanding of non-equilibrium states is moving forwards, but the fact that we cannot yet prove Fourier's law<sup>3</sup> (that heat conduction is inversely proportional to the length of the conductor) from first principles shows how far we still have to go.

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## Signal transduction

# Life-or-death decisions

John M. Kyriakis

In response to a protein that is linked to inflammation, cells either die or survive. Some molecular sleuthing has shed light on how the balance is tipped towards survival.

The cells of multicellular organisms are often forced to choose between living and dying. For example, immature T cells that react against the body's own proteins are made to commit cell 'suicide', a process known as programmed cell death or apoptosis. By contrast, immune cells that can fight an invading pathogen are stimulated to survive and proliferate — but only until the infection is cleared, at which point many of the now unnecessary cells also die by apoptosis. In the immune system and elsewhere, the protein tumour-necrosis factor (TNF) can signal both cell death and survival. But what determines whether a cell lives or dies in response to TNF? And is there any way in which the life and death signalling pathways can influence each other's output? Tang and colleagues<sup>1</sup> and De Smaele and co-workers<sup>2</sup> address these questions on pages 313 and 308 of this issue, with interesting results.

TNF is a cytokine — a protein that marshals and coordinates immune and inflammatory responses to infections or chronic stress. TNF exerts its effects by binding to its receptors, which are present on the surfaces of many different target cells. When occupied, these receptors recruit a cohort of intracellular 'adaptor' proteins, which

couple the receptors to intracellular signalling pathways. These pathways then organize and implement the cell's response (Fig. 1, overleaf). The so-called JNK and NF- $\kappa$ B signalling pathways, both of which consist of tiers of protein kinases (enzymes that add phosphate groups to target proteins), are pivotal in determining whether cells die or survive, respectively, in response to TNF<sup>3,4</sup>.

The JNKs are part of the evolutionarily conserved mitogen-activated protein kinase (MAPK) family, and are implicated in cell-death pathways stimulated by environmental stresses and TNF (Fig. 1)<sup>3,5,6</sup>. Once activated, JNK proteins can move from the cytoplasm of the cell into the nucleus. There, they phosphorylate and activate numerous transcription factors — proteins that control gene expression<sup>3</sup>. However, the exact mechanisms by which JNKs contribute to cell death are still unknown.

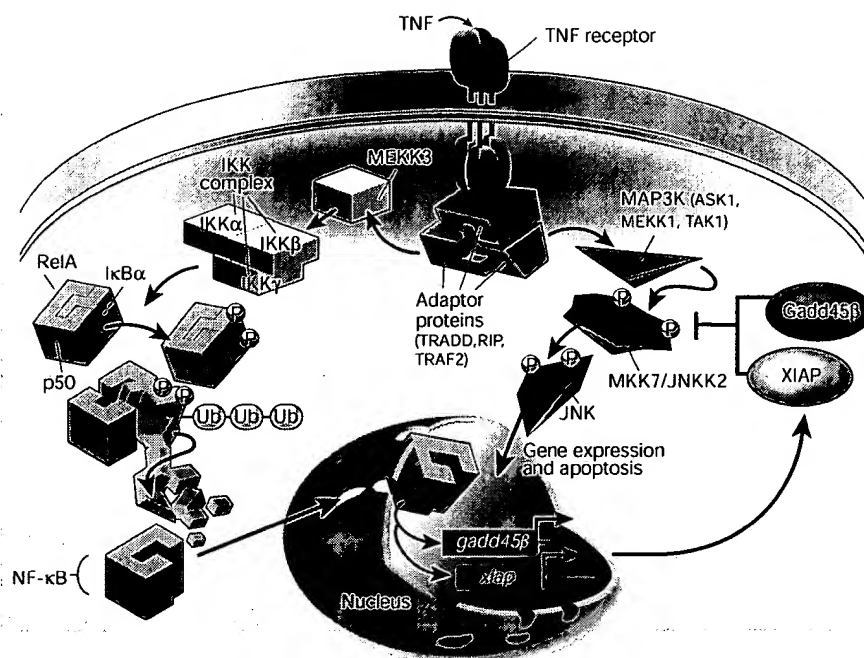
The survival pathway that is recruited by TNF requires transcription factors of the NF- $\kappa$ B/Rel family (Fig. 1). In resting cells, NF- $\kappa$ B is held captive outside the nucleus — away from its target genes — by proteins of the I $\kappa$ B family. Binding of TNF to its receptors initiates a signalling pathway that culminates in the phosphorylation of the I $\kappa$ Bs by the trimeric I $\kappa$ B kinase (IKK) complex.

Phosphorylation marks I $\kappa$ Bs for degradation; this liberates NF- $\kappa$ B, which then heads for the nucleus to activate a programme of gene expression<sup>4</sup>. NF- $\kappa$ B enhances cell survival by switching on genes that dampen pro-apoptotic signals; however, only a few of these genes have been identified<sup>4,7</sup>.

How a cell responds to TNF can vary, and depends on the physiological context, and so it is important to understand the molecular basis on which the cell chooses between life and death. Tang *et al.*<sup>1</sup> and De Smaele *et al.*<sup>2</sup> were investigating in detail how the NF- $\kappa$ B pathway protects cells from apoptosis. In particular, they wanted to identify additional mechanisms and target genes involved, and to determine how these genes interact with the cell-death signalling machinery. Both teams found that one of the ways in which NF- $\kappa$ B protects cells from apoptosis is by substantially blunting the JNK pathway. The activation of JNK in response to TNF was much stronger and more prolonged when the NF- $\kappa$ B pathway was made inoperative, as in cells lacking RelA (a subunit of NF- $\kappa$ B) or IKK $\beta$  (the subunit of the IKK complex whose activity is dependent on TNF), or in cells expressing a mutant I $\kappa$ B that could not be degraded<sup>1,2</sup>. These effects were specific to the activation of JNK by TNF: the activation of two close relatives of JNK was unimpaired, as was the activation of JNK by interleukin-1, another cytokine involved in inducing inflammation<sup>1</sup>.

Given that NF- $\kappa$ B controls gene expression, it seemed reasonable to suppose that NF- $\kappa$ B might suppress the JNK pathway by activating genes that encode inhibitors of JNK activation. Tang *et al.* and De Smaele *et al.* each identified a different JNK-inhibitory gene. De Smaele *et al.*<sup>2</sup> searched for protective genes expressed specifically in cells with a functional NF- $\kappa$ B pathway, not in cells in which this pathway is disrupted. They identified *gadd45 $\beta$*  — one of a family of genes that encode proteins associated with cell-cycle control and DNA repair<sup>8,9</sup>. They found<sup>2</sup> that the Gadd45 $\beta$  protein was expressed in response to TNF in an NF- $\kappa$ B-dependent way, and reduced the activation of JNK. They also found that when *gadd45 $\beta$*  expression was disrupted, JNK was more strongly activated. Tang *et al.*<sup>1</sup> identified *xiap* as a second gene that is switched on by TNF in an NF- $\kappa$ B-dependent manner. They also showed that forced expression of low levels of *xiap* reduced activation of JNK by TNF.

Just how the Gadd45 $\beta$  and XIAP proteins blunt the activation of JNKs and suppress apoptosis is unclear. Curiously, each has been found before in connection with the JNKs — but as possible JNK activators. The Gadd45 proteins, expressed in response to DNA damage, can associate with and, in the case of Gadd45 $\gamma$ , stimulate a protein kinase that can activate JNK<sup>9</sup>. So it was suggested<sup>9</sup> — albeit controversially<sup>10,11</sup> — that



**Figure 1** Crosstalk between life and death signalling pathways. The inflammatory molecule tumour-necrosis factor (TNF) recruits two pathways, which culminate in the activation of JNK or NF- $\kappa$ B. Both begin (top) with the binding of TNF to its receptors, which are coupled to downstream events by adaptor proteins<sup>3</sup>. Left, in the NF- $\kappa$ B pathway, the next step<sup>14</sup> is the activation of the enzyme MEKK3. This in turn activates the IKK complex, which adds phosphate groups (P) to I $\kappa$ B $\alpha$ , ensuring that this protein is modified with ubiquitin groups (Ub) — a prerequisite for proteolytic destruction. NF- $\kappa$ B is then free to move to the nucleus, where it activates target genes. Right, in the JNK pathway, a cascade of protein kinases leads to the activation of JNKs, some of which move to the nucleus and influence the expression of a different set of genes. Tang *et al.*<sup>1</sup> and De Smaele *et al.*<sup>2</sup> have found that NF- $\kappa$ B induces the expression of *gadd45 $\beta$*  and *xiap*, which blunt the JNK pathway and so promote cell survival.

the Gadd45 proteins couple signalling pathways that are recruited by DNA damage to JNK activators. Moreover, in T lymphocytes, disruption of *gadd45 $\gamma$*  dampens the activation of JNK by the T-cell antigen receptor<sup>12</sup>. However, these results pertain primarily to Gadd45 $\gamma$ , not Gadd45 $\beta$ .

Meanwhile, XIAP was identified as a 'bridging' protein that indirectly couples receptors for bone morphogenetic proteins (a group of cytokines important in development) to the activation of JNK and other MAPKs<sup>3</sup>. How, then, might it blunt the activation of JNK by TNF? Perhaps, when bone morphogenetic protein is absent, XIAP instead sequesters elements that are needed for TNF to activate JNKs. One such element could be TAK1, a protein kinase that associates indirectly with XIAP and has been implicated<sup>13</sup> in the activation of both IKKs and JNK by interleukin-1. Yet NF- $\kappa$ B does not affect the recruitment of JNK in response to interleukin-1. Further studies are clearly needed.

How might these new results<sup>1,2</sup> fit into a dynamic, context-dependent model for TNF-induced signalling? Under different physiological circumstances, different adaptor proteins could be recruited to TNF receptors, favouring the activation of NF- $\kappa$ B or JNK and so influencing the contribution of the 'crosstalk' effect. For example, apoptosis-

signal-regulating kinase-1 is a protein kinase required for the sustained activation of JNK by TNF in fibroblast cells<sup>9</sup>. Moreover, prolonged activation of NF- $\kappa$ B triggers the expression of its inhibitor, I $\kappa$ B, reducing further activation<sup>4</sup>. The contribution of these two effects might ensure that long-term exposure to TNF preferentially activates JNK-dependent apoptosis. It will be interesting to see how cell- and context-specific signalling elements are integrated with the newly discovered crosstalk between these general pathways of life and death signalling.

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## Daedalus

### Slippery light aircraft

The drag on an aeroplane, says Daedalus, is largely due to the impact of its 'wetted surface' with still air. Each colliding air molecule is momentarily adsorbed and later re-emitted at some unpredictable angle, running away with energy. The unwetted ideal, in which each molecule bounces off directly like light from a mirror, almost never happens. A rusty wing, resisting the attacking oxygen, seems worth trying; but this still leaves the nitrogen in the air. In any case, most aircraft wings are aluminium. This is superficially oxidized to transparent aluminium oxide, but is still well wetted by oxygen in the air. Surface tension is well understood in liquids, but has been largely ignored in gases. Daedalus recalls the chemical notion of anti-bonding, in which one molecule is repelled by another if the bond between them is raised by light or near-ultraviolet irradiation to an anti-bonding state.

Illuminated aircraft were actually tried during the Second World War to reduce the contrast between plane and sky as seen from the ground. Daedalus reckons that a non-wetted, anti-bonding plane should fly. DREADCO physicists are therefore fitting an aluminium plane with two sets of lights: one to raise the bond between aluminium oxide and oxygen to anti-bonding, the other to do the same for the bond between aluminium oxide and nitrogen. Such an aircraft would then remain untouched by the chief constituents of the air. Illuminated wings and fuselage should simply not be wetted.

Daedalus predicts a dramatic loss of drag. Only the windows would remain to contribute to the normal drag. The new slippery aircraft would require much less power. Even better, the propellers or the inlet fans of the jet engines could also be illuminated. The drag of these fast-moving elements would be cancelled as well, greatly increasing their efficiency. The whole plane should consume a small fraction of the usual power.

Aerodynamicists will at last be able to predict the behaviour of wings and fuselage simply. All the problems of the boundary layer will vanish: there won't be one. Molecules will bounce off an unwetted aircraft perfectly. Daedalus reckons that there should be a great saving of fuel, helping to reduce carbon dioxide emissions. Sadly, the usual painted logos must be abandoned, otherwise more light wavelengths will have to be added to reverse the bonds between the paint and the air's oxygen and nitrogen. **David Jones**

# The Dorsoventral Regulatory Gene Cassette *spätzle/Toll/cactus* Controls the Potent Antifungal Response in *Drosophila* Adults

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## Summary

The cytokine-induced activation cascade of NF- $\kappa$ B in mammals and the activation of the morphogen dorsal in *Drosophila* embryos show striking structural and functional similarities (Toll/IL-1, Cactus/I- $\kappa$ B, and dorsal/NF- $\kappa$ B). Here we demonstrate that these parallels extend to the immune response of *Drosophila*. In particular, the intracellular components of the dorsoventral signaling pathway (except for dorsal) and the extracellular Toll ligand, *spätzle*, control expression of the antifungal peptide gene *drosomycin* in adults. We also show that mutations in the Toll signaling pathway dramatically reduce survival after fungal infection. Antibacterial genes are induced either by a distinct pathway involving the *immune deficiency* gene (*imd*) or by combined activation of both *imd* and dorsoventral pathways.

## Introduction

Insects have developed an efficient host defense against microorganisms that relies on three major responses: proteolytic cascades, namely the coagulation and the phenoloxidase cascades, which are immediately induced upon injury; phagocytosis and encapsulation of invading microorganisms by circulating blood cells; and the rapid and transient synthesis, following injury, of a battery of potent antimicrobial peptides (reviewed by Hultmark, 1993; Boman, 1995; Hoffmann, 1995). These molecules are primarily produced by the fat body, a functional analog of the mammalian liver, and are secreted into the blood. Recent studies have identified in *Drosophila* two functionally distinct classes of inducible antimicrobial molecules: antibacterial peptides, namely cecropins (Kylsten et al., 1990; Tryselius et al., 1992), dipterin (Wicker et al., 1990), drosocin (Bulet et al., 1993), attacin (Asling et al., 1995), insect defensin (Dimarcq et al., 1994), and an antifungal peptide, *drosomycin* (Fehlbaum et al., 1994).

The elucidation of the control mechanisms that lead to the rapid synthesis of the antimicrobial peptides after septic injury is one of the challenges in this field. We have recently described a recessive mutation, *immune deficiency* (*imd*), which impairs the inducibility of all genes encoding antibacterial peptides during the immune response of *Drosophila*. This gene has not yet been cloned but, interestingly, the antifungal peptide *drosomycin* remains fully inducible in homozygous *imd*

mutants, a result which points to the existence of different pathways leading to expression of the antifungal and antibacterial peptide genes (Lemaitre et al., 1995a).

We have undertaken the characterization of these pathways. For this, we were guided by the parallels that exist between the cytokine-induced activation cascade of NF- $\kappa$ B during the inflammatory response in mammals (reviewed by Siebenlist et al., 1994; Baeuerle and Henkel, 1994) and the activation of the morphogen dorsal during embryonic dorsoventral patterning in *Drosophila* (reviewed by Wasserman, 1993; Morisato and Anderson, 1995). These parallels can be summarized as follows (see references above): first, NF- $\kappa$ B and dorsal (*dl*; Steward, 1987) are members of the Rel family of rapidly inducible transactivators; second, NF- $\kappa$ B and *dl* are retained in the cytoplasm by the inhibitory proteins I- $\kappa$ B and cactus, respectively (cact; Kidd, 1992; Geisler et al., 1992), which are structurally related; third, once NF- $\kappa$ B and dorsal are released from I- $\kappa$ B or cactus, they translocate into the nucleus and interact with target promoters via closely related  $\kappa$ B-binding sites; fourth, dissociation of the NF- $\kappa$ B-I- $\kappa$ B and *dl*-cact complexes is induced in a signal-dependent manner via transmembrane receptors: significantly, two of the well-established receptors, the interleukin-1 (IL-1) receptor which mediates nuclear transport of NF- $\kappa$ B in mammalian lymphocytes and the Toll receptor (Tl; Hashimoto et al., 1988) which mediates nuclear import of *dl*, share sequence homology in their cytoplasmic domains (Gay and Keith, 1991; Heguy et al., 1992); and, fifth, signaling through these two receptors may involve protein kinases such as interleukin-receptor associated kinase (IRAK; Cao et al., 1996) and the *pelle* gene product (*pil*; Shelton and Wasserman, 1993), which also share sequence similarities. The idea that these parallels could be extended to the immune response of *Drosophila* became attractive when  $\kappa$ B-binding sites were observed in the promoters of the genes encoding antibacterial peptides (Sun et al., 1991; reviewed by Hultmark, 1993). These sites were shown to play a pivotal role in the bacterial induction of the dipterin and the cecropin genes (Kappler et al., 1993; Engström et al., 1993). In addition, the immune responsive fat body cells of *Drosophila* were found to express the two genes encoding Rel proteins: dorsal, initially identified as the dorsoventral morphogen (Reichhart et al., 1993), and Dif (for dorsal-related immunity factor; Ip et al., 1993). Following an immune challenge, both proteins undergo a rapid translocation from the cytoplasm to the nucleus in these cells, and the *dl* and *dif* genes are induced (Ip et al., 1993; Reichhart et al., 1993; Petersen et al., 1995). Interestingly, in *77* gain-of-function mutants in which the *dl* pathway is activated in a signal-independent fashion, both proteins are constitutively present in the nucleus of the fat body cells (Ip et al., 1993; Lemaitre et al., 1995b). Intriguingly though, neither the dipterin nor the cecropin gene is constitutively expressed in these mutants (Lemaitre et al., 1995b).

In the present study, we have analyzed the expression of both the antifungal and antibacterial peptide genes

in strains carrying mutations that affect dorsoventral patterning in the embryo. We show that the embryonic regulatory pathway comprising the gene products between the *Tl* ligand *spätzle* (*spz*; Morisato and Anderson, 1994; Schneider et al., 1994) and *cact*, but not the genes acting upstream or downstream, play a major role in the control of the antifungal peptide gene *drosomycin*. Our results confirm the existence of basic differences in the control of expression of the antifungal versus the antibacterial peptides. They also point to unexpected discrepancies in the control of expression of some of the antibacterial genes in adults: while the expression of all these genes was dependent on the product of the *imd* gene (Lemaître et al., 1995a), the full induction of cecropin A genes, but not that of dipterin, additionally required components of the dorsoventral pathway. We show furthermore that flies mutant for these components have a dramatically reduced resistance to fungal, but not to bacterial, infection. Flies mutant for both the *imd* and the *Tl* signaling pathways fail to express to a significant extent any of the antimicrobial genes and rapidly succumb to either fungal or bacterial infections, indicating that these pathways are essential for antimicrobial resistance in *Drosophila*. Consistent with our inference that the dorsoventral regulatory gene cassette extending from *spz* to *cact* is involved in the antimicrobial response of *Drosophila* adults, we show that these genes are all expressed in adults and that their expression is up-regulated by immune challenge.

## Results

The results reported in this study were obtained with adult *Drosophila*. Two types of mutants were analyzed: first, strains carrying strong loss-of-function mutations of *gastrulation defective* (*gd*), *snake* (*snk*; DeLotto and Spierer, 1986), *easter* (*ea*; Chasan and Anderson, 1989), *spz*, *Tl*, *tube* (*tub*; Letsou et al., 1991), *pil*, and *dl*, which are known to block the dorsoventral signaling pathway, leading to completely dorsalized embryos; second, gain-of-function mutations in *Tl* (*Tl<sup>D</sup>*) and loss-of-function mutations in *cact* that are strongly ventralizing (see references in Experimental Procedures). Dorsalizing mutants were challenged by an injection of bacteria, which induces both the antifungal and the antibacterial genes (Fehlbaum et al., 1994; Lemaître et al., 1995a), whereas ventralizing mutants were analyzed in the absence of challenge. We have examined antimicrobial gene expression by Northern blot analysis and followed the survival rate in dorsoventral and *imd* mutants infected with either bacteria or fungi. We have also studied the expression of the dorsoventral signaling genes after bacterial challenge.

### Components of the Dorsoventral Pathway Control the *drosomycin* Gene

A first striking result was obtained when *drosomycin* expression was analyzed in *Tl<sup>D</sup>* (*Tl<sup>10b</sup>* and *Tl<sup>9Q</sup>*) gain-of-function and *cact*-deficient adults, in which the antifungal gene was found to be constitutively expressed in the absence of immune challenge. The level of expression was similar to that induced by bacterial challenge

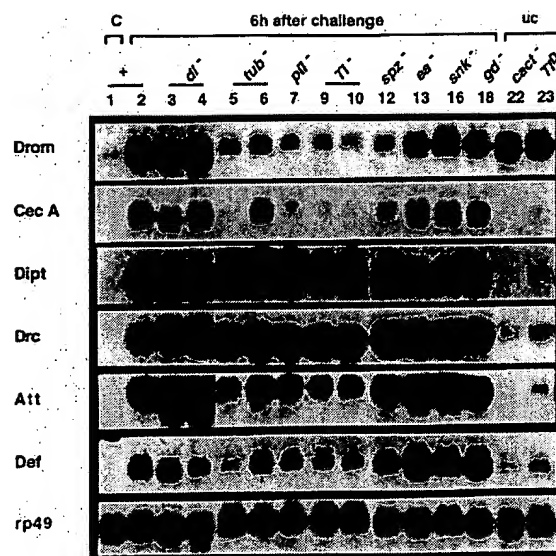


Figure 1. Transcriptional Profiles of Antimicrobial Genes in Wild-Type and Dorsoventral Mutant Adults

A representative Northern blot of total RNA extracted from bacteria-challenged wild-type and mutant adults. The blot was successively hybridized with the following nick-translated cDNA probes: *Drom*, *drosomycin*; *Cec A*, cecropin A1; *Dipt*, dipterin; *Drc*, drosocin; *Att*, attacin; *Def*, defensin; *rp49*. C, control; uc, unchallenged. Lanes are as follows: 1 and 2, Oregon<sup>R</sup> (*Or<sup>R</sup>*); 3, *dl<sup>1</sup>/dl<sup>1</sup>*; 4, *dl<sup>1</sup>/Df(dl)*; 5, *tub<sup>238</sup>/tub<sup>118</sup>*; 6, *tub<sup>2</sup>/tub<sup>118</sup>*; 7, *pil<sup>1078</sup>/pil<sup>21</sup>*; 8, *Tl<sup>632</sup>/Tl<sup>1-23A</sup>* (29°C); 9, *Tl<sup>444</sup>/Tl<sup>POURE</sup>* (29°C); 10, *spz<sup>mm</sup>/spz<sup>mm</sup>*; 11, *ea<sup>1</sup>/ea<sup>1</sup>*; 12, *ea<sup>1</sup>/ea<sup>1</sup>*; 13, *ea<sup>1</sup>/ea<sup>1</sup>*; 14, *snk<sup>1073</sup>/snk<sup>1073</sup>*; 15, *gd<sup>1</sup>/gd<sup>1</sup>*; 16, *gd<sup>1</sup>/gd<sup>1</sup>*; 17, *cact<sup>42</sup>/cact<sup>42</sup>*; 18, *Tl<sup>10b</sup>/+*; 19, *Tl<sup>10b</sup>/+*; 20, *Tl<sup>10b</sup>/+*; 21, *Tl<sup>10b</sup>/+*; 22, *Tl<sup>10b</sup>/+*; 23, *Tl<sup>10b</sup>/+*.

in wild-type adults (Figure 1; for a more detailed analysis, see Figure 2). To ascertain that *drosomycin* was actually synthesized in *Tl<sup>D</sup>* mutants, we extracted the peptide from these insects, purified it by high pressure liquid chromatography, and quantified it by optical density measurements with reference to standard *drosomycin* (Fehlbaum et al., 1994). The following results were obtained for 400 adult males: unchallenged wild-type adults, no detectable *drosomycin*; bacteria-challenged wild-type adults, 7.6 nmol; unchallenged *Tl<sup>10b</sup>* mutants, 5 nmol. These results corroborate the data obtained by RNA measurements on the constitutive expression of the *drosomycin* gene in *Tl<sup>10b</sup>* mutants and demonstrate that in these experiments the peptide was produced when the gene was transcribed.

When *spz*-, *Tl*-, *tub*-, and *pil*-deficient mutant adults were bacteria challenged, the level of induction of the *drosomycin* gene was significantly lower (4- to 5-fold) than in wild-type insects (Figures 1 and 2). In agreement with these results, bacteria-challenged *Tl* adults contained only 0.9 nmol of peptide per 400 insects (i.e., 12% of the value for challenged wild type). We do not attribute the lower level of induction to the general genetic backgrounds of these mutants, since two different alleles were tested for each gene (Figure 2) and flies carrying only the genetic markers showed a wild-type level of induction for the *drosomycin* gene after challenge (data not shown; see Experimental Procedures for the list of markers). As reported for embryonic development, one of the *Tl* alleles that we tested (*Tl<sup>632</sup>*) was also temperature sensitive for the expression of the *drosomycin* gene. The immune-induced expression of this

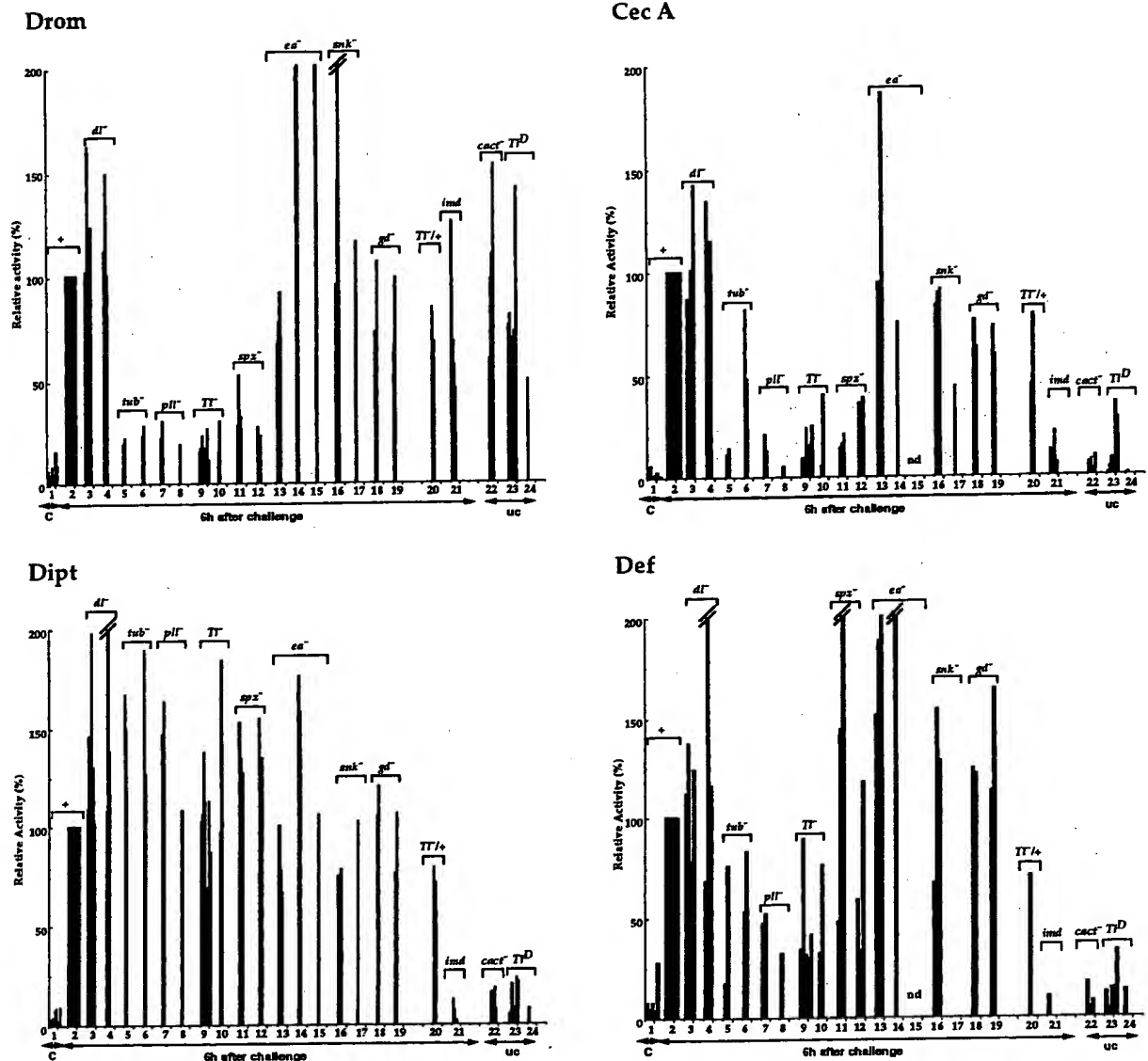


Figure 2. Expression of Antimicrobial Genes in Wild-Type and Dorsoventral Mutant Adults

The signals on several Northern blots similar to that presented in Figure 1 were quantified by a Bio-Imager system. In each experiment, the signals of the immune gene expression were normalized with the corresponding value of the *rp49* signal. The levels of expression in 6 hr bacteria-challenged wild-type adults were standardized as 100 and the results are given as relative activity (percent). Each bar corresponds to an independent experiment. Analyses of Northern blots for drosomycin (Drom), cecropin A1 (Cec A), dipterocin (Dipt), and defensin (Def) gene expression are presented. Results obtained for drosocin and attacin were similar to those for dipterocin and defensin, respectively (data not shown). C, control; uc, unchallenged. Lanes are as follows: 1 and 2, *Or*<sup>R</sup>; 3, *dl*<sup>1</sup>/*dl*<sup>1</sup>; 4, *dl*<sup>1</sup>/*Df*(*dl*); 5, *tub*<sup>230</sup>/*tub*<sup>118</sup>; 6, *tub*<sup>2</sup>/*tub*<sup>118</sup>; 7, *pII*<sup>1078</sup>/*pII*<sup>1</sup>; 8, *pII*<sup>1078</sup>/*pII*<sup>106</sup>; 9, *pII*<sup>632</sup>/*TI*<sup>1-RXA</sup> (29°C); 10, *TI*<sup>144</sup>/*TI*<sup>POURE</sup> (29°C); 11, *spz*<sup>197</sup>/*spz*<sup>197</sup>; 12, *spz*<sup>197</sup>/*spz*<sup>197</sup>; 13, *ea*<sup>1</sup>/*ea*<sup>1</sup>; 14, *ea*<sup>818</sup>/*ea*<sup>818</sup> (29°C); 15, *ea*<sup>1</sup>/*ea*<sup>2</sup>; 16, *snk*<sup>1073</sup>/*snk*<sup>1073</sup>; 17, *snk*<sup>1073</sup>/*snk*<sup>229</sup>; 18, *gd*<sup>1</sup>/*gd*<sup>1</sup>; 19, *gd*<sup>1</sup>/*gd*<sup>1</sup>; 20, *TI*<sup>1-RXA</sup>/*+*; 21, *imd*/*imd*; 22, *cact*<sup>12</sup>/*cact*<sup>12</sup>; 23, *TI*<sup>106</sup>/*+*; 24, *TP*<sup>10</sup>/*+*.

gene was indeed more strongly affected in *TI*-deficient mutants when the adults were placed at 29°C, at which temperature the level of inducibility relative to wild type was 15%, as compared with 40% at 18°C. We also noted that *TI* behaved as a recessive mutant, since heterozygous *TI*<sup>+</sup>/*+* adults exhibited a close to wild-type level of expression of the drosomycin gene (Figure 2).

In contrast with adults deficient in *spz*, *TI*, *tub* and *pII*, mutants in the *gd*, *snk* and *ea* genes, which lie upstream in the dorsoventral patterning cascade, show a wild-type response to bacterial challenge (Figures 1 and 2). The *ea* and *snk* genes encode serine proteases (DeLotto

and Spierer, 1986; Chasan and Anderson, 1989), that are involved in processing of the *spz* gene product. Since the alleles of *ea* and *snk* used in our study are point mutations in the catalytic site, which result in non-functional proteases (Jin and Anderson, 1990; Smith et al., 1994), our results indicate that neither *ea* nor *snk* is required for the control of drosomycin gene expression. Finally, in *dl* mutants, drosomycin gene induction was not affected.

Since the preceding data were based on a single time-point (6 hr postchallenge), we wondered whether in mutants in the dorsoventral regulatory pathway the time



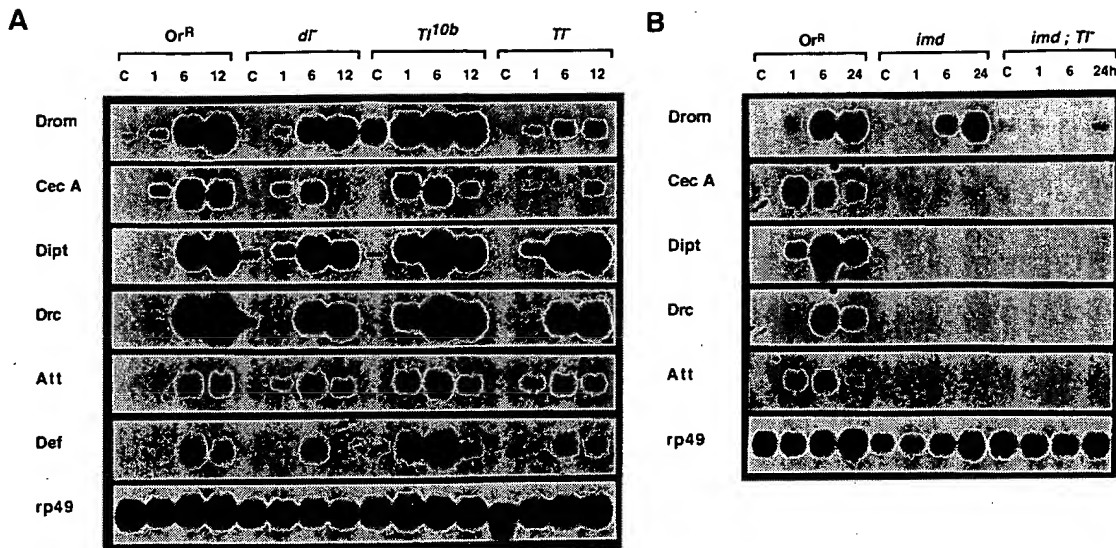


Figure 3. Time Course Analysis of Antimicrobial Gene Expression in Wild-Type and Mutant Adults

Northern blot analysis of total RNA extracted at different time intervals after challenge (as indicated). Flies were bacteria challenged and processed together. (A) and (B) were obtained separately.

(A) *Or<sup>R</sup>*, *dl<sup>1</sup>/dl<sup>1</sup>* (*dl<sup>-</sup>*), *Tl<sup>10b</sup>/+* (*Tl<sup>10b</sup>*), *Tl<sup>632</sup>/Tl<sup>1-ROA</sup>* (*Tl<sup>-</sup>*).

(B) *Or<sup>R</sup>*, *imd/imd* (*imd*) and *imd/imd; Tl<sup>632</sup>/Tl<sup>POURE</sup>* (*imd; Tl<sup>-</sup>*).

course of drosomycin might also be affected (Figure 3A). These experiments were restricted to gain-of-function and loss-of-function *Tl* mutants and to *dl* mutants. No striking discrepancies were observed, and the results basically confirm the data presented above in that drosomycin induction is low in a *Tl*-deficient background, similar to that of wild-type in *dl* mutants, and the gene is constitutively expressed in *Tl<sup>10b</sup>* mutants. Interestingly, the level of expression was increased beyond the constitutive level after bacterial challenge.

Altogether our results indicate that the embryonic regulatory pathway, comprising the gene products between *spz* and *cact* but not the genes acting upstream or downstream, is involved in the induction of the drosomycin gene in adults.

#### Distinct Pathways Control the Expression of the Antibacterial Genes in Adults

The Northern blots used above were repeatedly dehybridized and successively probed with cecropin A1, diptericin, drosocin, attacin, and defensin cDNAs. We first observed that, in contrast with the drosomycin gene, no strong constitutive expression occurred for any of the antibacterial peptide genes in gain-of-function *Tl* and *cact*-deficient mutant adults (Figures 1 and 2). However, the level of constitutive expression of all antibacterial genes in these mutants was higher than the basal level (roughly 2- to 5-fold higher; Figure 2).

The induction of the cecropin A genes (two cecropin A genes, A1 and A2, differ in their nucleotide sequence but code for the same protein; Kylsten et al., 1990) by bacterial challenge was markedly reduced in adults deficient for *spz*, *Tl*, *tub*, and *pII*, the level of transcripts being approximately 4-fold lower than in wild-type flies. In contrast, cecropin A gene induction was not affected in mutants deficient in *gd*, *snk*, *ea*, or *dl*. Note that for

the allelic combination *tub<sup>3</sup>/tub<sup>118</sup>*, the inducibility of the cecropin A genes was only slightly affected, in contrast with the *tub<sup>238</sup>/tub<sup>118</sup>* background (Figures 1 and 2). At present we have no explanation for this discrepancy. However, the observation that the inducibility of the drosomycin gene is altered in both *tub* backgrounds tested suggests that the expression of cecropin A is less tightly controlled by the dorsoventral pathway than the antifungal peptide gene. Overall, the pattern of response of the cecropin A genes paralleled that of the drosomycin gene, except that cecropin A was not constitutively expressed in *cact*-deficient and *Tl<sup>p</sup>* gain-of-function mutants.

In contrast with the cecropin A genes, the diptericin and the drosocin genes remained fully inducible in all the mutants tested above, including *spz*, *Tl*, *tub*, and *pII*. The induction of the attacin and defensin genes was slightly weaker in *Tl*, *tub*, and *pII* mutants than in wild-type or *ea*, *snk*, and *dl* mutants, which behaved as wild type. Surprisingly, the inducibility of the last two genes was less altered in *spz* mutants than in *pII*-, *tub*-, and *Tl*-deficient flies. In general, the pattern of expression of these genes was intermediate between that of the cecropin A genes and the diptericin-drosocin group.

Experiments analyzing the time course of the induction of antibacterial peptide genes reinforced these results. In *Tl*-deficient adults, the cecropin A and, to a lesser extent, attacin and defensin genes were only minimally inducible (Figure 3A), in contrast with the diptericin and drosocin genes, which remained fully inducible in this context. No marked constitutive expression was observed in *Tl<sup>p</sup>* gain-of-function mutants which, interestingly, exhibited a more rapid induction of all antibacterial genes after bacterial challenge (compare *Tl<sup>10b</sup>* and wild-type adults 1 hr after bacterial challenge in Figure 3A). This last result, as well as the previous observation that



in *cact* and *Tl<sup>P</sup>* mutants antibacterial genes are expressed above basal level, indicates that the *Tl* pathway may slightly influence the expression of all the antibacterial genes (including diptericin and drosocin). As previously reported (Lemaître et al., 1995b), the induction of the antibacterial peptide genes in the *dl*-deficient adults was roughly similar to that in wild-type adults.

Altogether, these results strengthen the idea that genes encoding antibacterial peptides are regulated in a manner distinct from that of the gene encoding the antifungal peptide drosomycin. They also demonstrate that the antibacterial genes differ in their requirements for the dorsoventral pathway components. Cecropins A are the only genes that clearly require the *spz-cact* regulatory cassette for expression at the adult stage. However, constitutive activation of the *Tl* signaling pathway, as well as the absence of *cact*, are not sufficient to trigger a strong expression of these genes, in contrast with drosomycin.

#### The Induction of All Antimicrobial Genes Is Impaired in *imd;Tl* Double Mutant Flies

The drosomycin gene is only minimally expressed in *Tl*-deficient mutants, as shown above, and the expression of antibacterial peptide genes is impaired in *imd* mutants (Lemaître et al., 1995a). In the hope of generating flies with a severely depressed antimicrobial response, we have constructed lines homozygous for both the *imd* and the *Tl* mutations. Figure 3B shows that in these double mutants the inducibility of all antimicrobial genes by bacterial challenge was severely affected. No signal was detectable on Northern blots for any antibacterial gene for up to 24 hr following challenge, and only a faint signal was observed for drosomycin at 24 hr postchallenge.

#### Mutations That Affect the Synthesis of Antimicrobial Peptides Dramatically Lower the Resistance of Flies to Infection

We analyzed the survival rate of *imd*, *Tl* and *imd;Tl* mutant adults after fungal and bacterial infections. The insects received one of three treatments: pricking with a clean needle under nonsterile conditions (septic injury) to monitor the effect of injury and associated microorganisms from the environment; pricking with a needle dipped into a concentrated solution of spores of the fungus *Aspergillus fumigatus*; or pricking with a needle dipped into a concentrated solution of *Escherichia coli*. Northern blot analysis showed that these treatments all induced in wild-type adults the expression of the antibacterial and antifungal genes, the level of induction, however, being noticeably weaker for the first type of treatment (data not shown). The survival rate was followed over a 6 day period at 29° C. At this temperature, the susceptibility to microbial challenge is highest. The temperature sensitivity of infected adults most likely reflects the temperature-dependent growth of the microorganisms within the infected insects, as it affects all experimental flies regardless of their respective genotypes (data not shown).

As illustrated in Figure 4A, a septic injury did not noticeably affect wild-type adults or *imd* and *Tl* mutants. The survival of double mutants was more drastically affected, and only 40% had survived 6 days after septic injury.

Infections with *A. fumigatus*, a weak pathogen for insects (Vey and Götz, 1986), resulted in about one third mortality in the wild-type population (Figure 4B). Interestingly, the survival of *imd* mutants, in which only the synthesis of antibacterial peptides is impaired, was similar to that of wild type. In sharp contrast, *Tl*-deficient insects were dramatically affected by the fungus. All flies died after 2–3 days and death was clearly associated with uncontrolled fungal development, since flies were covered with fungal hyphae after their death, as illustrated in Figure 5.

The survival rates of wild-type flies and *Tl* mutants, which both produce antibacterial peptides, were not markedly affected after infection with *E. coli* (Figure 4C). Interestingly, bacterial counts performed on fly extracts over a 24 hr period following infection showed that no bacterial growth had occurred in these insects. The results were strikingly different in experiments with *imd* or *imd;Tl* mutants, in which only a few individuals survived after 3 days. In these mutants, bacterial growth was intense, and the number of bacteria was 1000 times higher than in wild-type or *Tl* flies at 24 hr postinfection (Figure 6).

We have extended the studies on the survival rates to the other members of the dorsoventral signaling pathway. *Tl<sup>P</sup>* and *cact* adults, in which the antifungal gene is constitutively expressed (see above), were not studied since they exhibit a lower viability even in the absence of challenge. Table 1 presents the percentage of survival 3 days after fungal or bacterial infection in flies mutated for each component of the pathway. Day 3 was chosen because the difference in survival rates in the preceding experiments (Figure 4) were more marked at this time-point. Our results show that flies deficient for *pll*, *tub*, and *spz* have a dramatically increased susceptibility to fungal infection, as was observed for *Tl*-deficient mutants. In contrast, in *dl* and *ea* mutants, the survival rate was similar to that of wild-type insects. None of these mutations had a marked effect on the survival rate for bacterial infections (Table 1), which is consistent with our observation that most of the antibacterial peptides remain fully inducible in these mutants (see above). These results underline the correlation between the impairment of drosomycin synthesis and the susceptibility to fungal infection. They also strengthen the inference that the *spz*, *Tl*, *pll*, and *tub*, but not the *ea* or *dl*, gene products are required for the antifungal host defense.

#### The Dorsoventral Genes Are Induced upon Immune Challenge

It is well established that the transcripts of the dorsoventral genes are present in ovaries and early embryos in relation to their role in dorsoventral patterning during early embryogenesis (reviewed by Morisato and Anderson, 1995). To lend further credit to the conclusion that these genes are involved in the immune response of adult insects, we have verified that they are actually

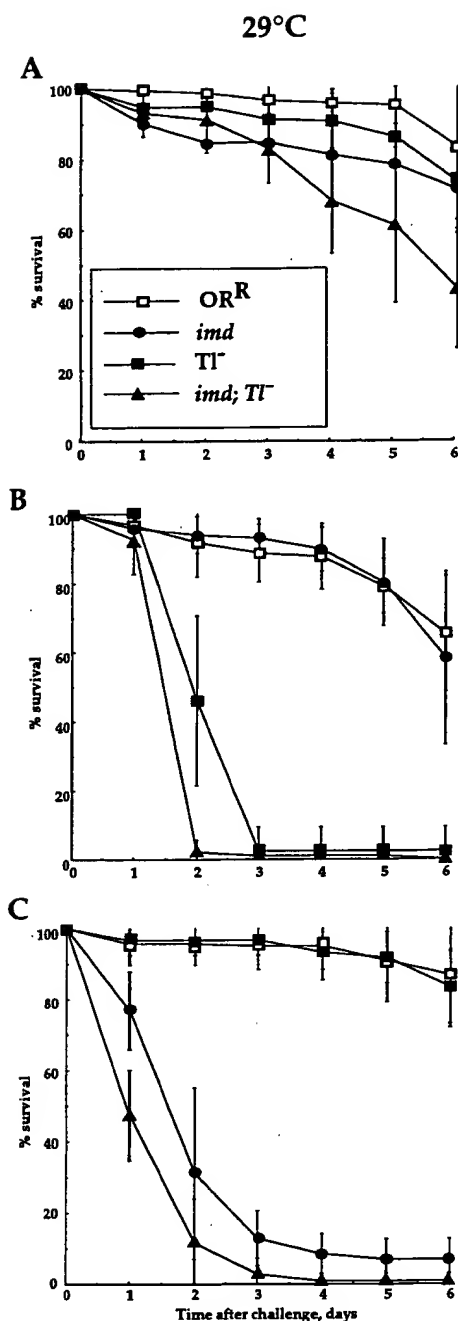


Figure 4. Survival of Wild-Type and Mutant Adults to Bacterial and Fungal Infections

The survival rates of infected *imd/imd* (*imd*); *TI<sup>632</sup>/TI<sup>1-RXA</sup>* (*TI*), *imd/imd*; *TI<sup>632</sup>/TI<sup>1-RXA</sup>* (*imd*; *TI*) mutants and wild-type (*Or<sup>R</sup>*) flies are presented with their confidence interval ( $p < 0.05$ ). Groups of 20 adults, aged 2–4 days, were pricked and transferred to a fresh vial every 4 days. The survival rates of untreated mutants were identical to those observed with wild type (>95% after 6 days; data not shown). At least five replicates were used for the determination of the survival rates. Adults were pricked with a needle previously dipped into either water (A), a concentrated solution of spores of *A. fumigatus* ( $10^6$  spores per milliliter) (B), or a concentrated solution of *E. coli* (C).



Figure 5. Germinating Hyphae of *A. fumigatus* on a Dead *Drosophila*. Scanning electron micrograph of a *Drosophila* adult that succumbed to infection by *A. fumigatus* and is covered with germinating hyphae (200× magnification).

expressed at this stage. We have successively probed in Northern blotting experiments poly(A) RNA prepared from control and immune-challenged adult males (to avoid interference with ovarian transcripts) with cDNAs of various genes of the dorsoventral cascade, plus cDNA encoding the Rel protein Dif. Interestingly, we observed that the *cact*, *dll*, *tub*, *TI*, and *spz* genes are expressed

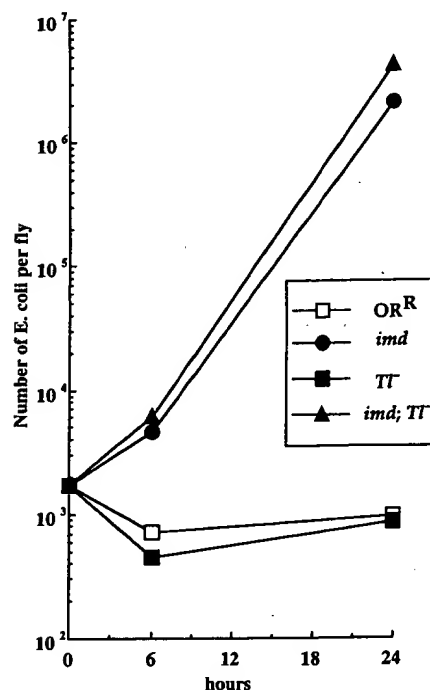


Figure 6. Bacterial Growth in Challenged Wild-Type and Mutant Adults Infected with *E. coli*

*imd/imd*, *TI<sup>632</sup>/TI<sup>1-RXA</sup>*, and *imd/imd*; *TI<sup>632</sup>/TI<sup>POURE</sup>* mutants and wild-type (*Or<sup>R</sup>*) flies were pricked with a needle dipped into a concentrated solution of *E. coli* carrying an ampicillin-resistant plasmid. Bacterial counts were obtained by plating an appropriate dilution of homogenates of five adults in phosphate-buffered saline on ampicillin medium. This experiment was repeated several times and yielded similar results.

Table 1. Survival of Dorsoventral Mutant Adults to Bacterial and Fungal Infections

Genotype Tested	Fungal Infection	Bacterial Infection
<i>Or<sup>R</sup></i>	89 (4.2; 9)	95 (5.3; 14)
<i>dl<sup>1</sup>/dl<sup>1</sup></i>	94 (4.3; 5)	92 (3.0; 6)
<i>pII<sup>178</sup>/pII<sup>21</sup></i>	4 (7.4; 5)	87 (8.5; 8)
<i>tub<sup>238</sup>/tub<sup>3</sup></i>	3 (5.3; 6)	71 (27; 4)
<i>TI<sup>632</sup>/TI<sup>1-RXA</sup></i>	8 (10.8; 8)	93 (6.6; 9)
<i>spz<sup>mi7</sup>/spz<sup>187</sup></i>	3 (5.6; 7)	84 (11; 9)
<i>ea<sup>1</sup>/ea<sup>2</sup></i>	98 (8.8; 5)	87 (5.7; 8)
<i>imd/imd</i>	93 (5.6; 5)	8 (7.4; 13)
<i>imd/imd; TI<sup>632</sup>/TI<sup>1-RXA</sup></i>	1 (2.3; 5)	3 (4.4; 6)

The survival rates are given in percentage, with standard deviation and the numbers of measurements in parentheses. The survival rate was measured 3 days after fungal (*A. fumigatus*) or bacterial (*E. coli*) infections. The conditions of these experiments were as described in Figure 4.

at a low level at this stage of development (Figure 7). More significantly in the present context, we noted that the transcription of all these genes (except for *tub*) was clearly up-regulated in response to immune challenge.

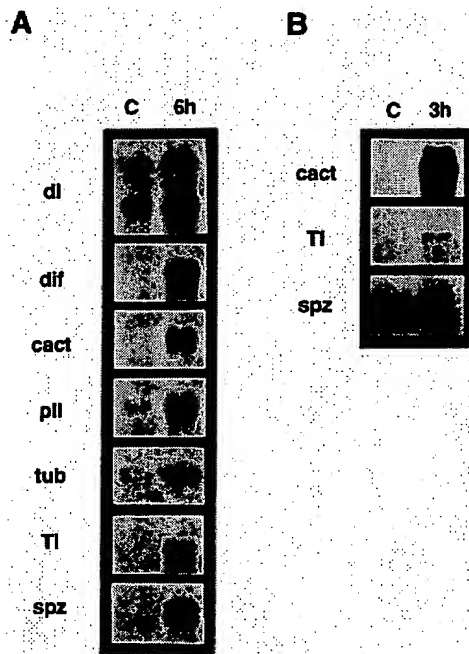


Figure 7. Expression of Dorsoventral Genes in Control and Challenged Adults

Aliquots containing 5  $\mu$ g of poly(A) RNA from uninduced (0 hr) or bacteria-challenged male adults were fractionated on an agarose-formaldehyde gel and subsequently hybridized with cDNA from the dorsoventral genes and from rp49 as a control. (A) and (B) were obtained from distinct experiments. Equivalent levels of rp49 signals were obtained for each experiment (data not shown). The sizes of the transcripts were similar to published values: *TI*, 5.3 kb (Hashimoto et al., 1988); *dif*, 2.8 kb (Ip et al., 1993); *dl*, 2.6 and 4.6 kb (Steward, 1987; Reichhart et al., 1993); *cact*, 2.2 kb (Geisler et al., 1992; Kidd, 1992); *pII*, 1.9 kb (Shelton and Wasserman, 1993); *tub*, 2 kb (Letsou et al., 1991); *spz*, 2.2 kb (Morisato and Anderson, 1994). The separating capacity of the gel was not sufficient to distinguish the two splicing isoforms of cactus RNA (Geisler et al., 1992; Kidd, 1992).

The overall level of inducibility 6 hr after bacterial challenge was estimated, by radioactive measurements and comparison with the reference rp49 transcript (data not shown), to be approximately 2- to 5-fold. Our results (Figure 7) also confirm previous data suggesting that the *dl* and *dif* genes are expressed at a basal level in male adults and that bacterial challenge increases their level of expression (Reichhart et al., 1993; Petersen et al., 1995). Antimicrobial peptide genes are mainly expressed in the fat body of adults, a thin tissue difficult to excise at this stage. However, a careful preparation of abdominal dorsal carcass of male flies allows extraction of predominantly adult fat body RNA with minor contaminations from epidermal and muscle RNA. As shown in Figure 7B, we observed that *cact*, *TI*, and *spz* genes are expressed in such preparations and that this expression is markedly up-regulated by bacterial challenge.

## Discussion

### Identification of a Pathway Mediating an Antifungal Response

Two major conclusions can be drawn from the analysis of the expression of the drosomycin gene in adults. First, in *TI* gain-of-function and *cact*-deficient mutants, the drosomycin gene is constitutively expressed and the level of expression is similar to that induced by bacterial challenge in wild-type adults. The activation of the *TI* signaling pathway is therefore sufficient for the induction of the antifungal gene. We have shown mutations in *Drosophila* to mimic an immune challenge, leading to the synthesis of an antimicrobial peptide. Second, loss of function in any of the genes extending in the dorsoventral regulatory cascade from *spz* to *pII* results in a markedly impaired induction of the drosomycin gene after bacterial challenge. These data lead us to conclude that the gene cassette comprising the intracellular part of the dorsoventral pathway (with the exception of *dl*), plus the extracellular component *spz*, is involved in the control of transcription of the drosomycin gene (see the model in Figure 8). Our inference is further substantiated by the observation that these genes are actually expressed at the adult stage (most probably in the fat body cells, to judge from the experiments with abdominal carcasses) and that their expression (with the exception of *tub*) is markedly up-regulated by immune challenge. The latter result further indicates that the components of the cascade are themselves controlled at the transcriptional level during the immune response.

Mutations in the *snk* and *ea* genes, which are upstream of *spz*, do not affect the inducibility of the drosomycin gene, indicating either that the *snk* and *ea* gene products are not involved in the induction of the drosomycin gene or that in their absence other gene products can fully substitute for their function. *snk* and *ea* encode serine proteases of the trypsin family (Chasan and Anderson, 1989; DeLotto and Spierer, 1986) and may serve to activate the *spz* gene product by proteolytic cleavage, enabling the processed protein to bind to the *TI* receptor (Morisato and Anderson, 1994; Schneider et al., 1994).

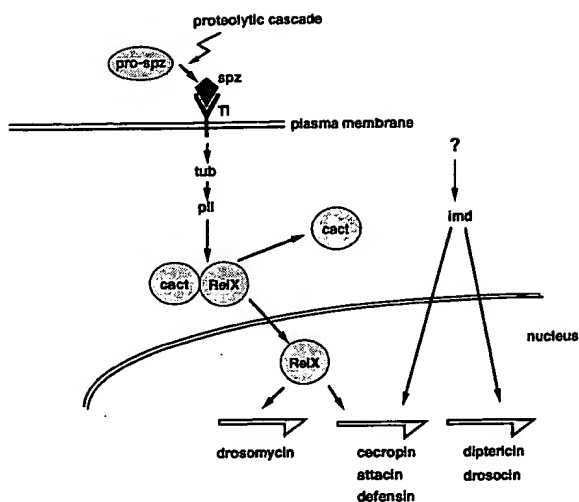


Figure 8. Model for the Control of Expression of Genes Encoding Antimicrobial Peptides in the *Drosophila* Fat Body of Adults

Two distinct pathways activate the expression of antimicrobial peptide genes in adults. Drosomycin is induced via a Rel protein (possibly Dif or an as yet unidentified protein, but not Df), which is retained in the cytoplasm of the fat body by cact. The dissociation of the cact-RelX complex is mediated by the intracellular *Tl*, *tub*, and *p11* gene products. By analogy with the embryonic system in which spz is present in the perivitelline fluid (reviewed by Morisato and Anderson, 1995), it is proposed that spz is present in the hemolymph, is processed by a protease of a proteolytic cascade induced upon septic injury (e.g., coagulation or phenoloxidase cascades), and acts as a ligand to activate the *Tl* receptor. The *imd* gene product has not been characterized and its precise role remains an open question. All the genes encoding antibacterial peptides require the *imd* gene product for their induction. In contrast with dipterecin and drosocin, the full induction of the cecropin A, defensin, and attacin genes also depends on the *Tl* pathway. We leave open the question of whether these two regulatory pathways act on the same or distinct transcriptional activator(s).

An attractive hypothesis is that the *Drosophila* proteolytic cascades initiated by injury, which lead to coagulation or melanization, activate as yet unidentified proteases, different from *snk* and *ea*, which cleave the spz protein to its functional form. The observation that serine proteases of the horseshoe crab clotting cascade share sequence similarities with the *ea* serine protease lends some credit to this hypothesis (reviewed by Hecht and Anderson, 1992; Iwanaga, 1993). In the embryonic system, spz is present in the perivitelline space, and we speculate that during the immune response spz is present in the hemolymph and plays a cytokine-like role (Figure 8). The involvement of the *spz* gene in the immune response of adults was unexpected since, in contrast with *cact*, *p11*, *tub*, and *Tl* (Gertulla et al., 1988; Roth et al., 1991; Hecht and Anderson, 1993), the *spz* mutant shows no striking zygotic phenotype.

The partner of *cact* in the drosomycin induction pathway has not yet been identified. The hypothesis that this partner is a member of the Rel family is consistent with the observation that the upstream sequences of the drosomycin gene contain several  $\kappa$ B-related binding motifs (L. M., unpublished data). Drosomycin is fully inducible in *dl*-deficient mutants, implying that *dl* does not play a key role in drosomycin induction. Dif may be

a better candidate. Indeed, this Rel protein has also been shown to bind in vitro to the cact protein (Tatei and Levine, 1995). The candidacy of Dif is further supported by the observation that in transfection experiments this protein is a more powerful transactivator for cecropin A1 gene than for dipterecin gene expression (Petersen et al., 1995; Gross et al., 1996). This result is in agreement with our present data showing that the induction of cecropin A, but not that of dipterecin, requires the dorsoventral gene cascade. To date, no mutants for Dif are available and we have to leave open the question of the nature of the transactivator acting on the drosomycin gene.

A preliminary analysis of the regulation of the antimicrobial peptide genes in larvae indicates that the dorsoventral components are also involved, but point to additional regulatory mechanisms at this stage (unpublished data).

#### The *Tl* and *imd* Signaling Pathways Are Both Required for a Full Antibacterial Response in Adults

The antibacterial response of *Drosophila* involves the coordinate expression of at least five genes encoding bactericidal peptides. Preliminary data point to the existence of several additional inducible antibacterial peptides in this species (P. Bulet, personal communication). The presence of  $\kappa$ B-binding sites in the promoters of all antibacterial genes (reviewed by Hultmark, 1993) and the requirement for the *imd* gene product (Lemaitre et al., 1995a) suggested that a common regulatory mechanism controls the expression of all these genes. The present study points to a more complex picture in adults (see the model in Figure 8). In addition to a functional pathway involving the *imd* gene product, the cecropin A genes, and to a lesser extent the attacin and insect defensin genes, also require the products of the *spz*, *Tl*, *tub*, and *p11* gene cassette, which is clearly dispensable for the induction of the dipterecin and the drosocin genes. In the tumorous blood cell line *mbn-2*, overexpression of *Tl<sup>100</sup>* had been observed to increase transcription of a cecropin A1 reporter gene, which was further stimulated by lipopolysaccharide, a result which also suggests that two cascades exist in the control of this immune gene (Rosetto et al., 1995). We observed that in *Tl<sup>p</sup>* gain-of-function and *cact*-deficient mutants, neither the cecropin A nor the attacin and insect defensin genes showed a marked constitutive expression, which indicates that the induction of this group of genes requires the concomitant activation of both pathways (Figure 8). Similarly, data obtained with knockout mice have recently shown that some of the NF- $\kappa$ B-responsive genes are constitutively expressed in mice deficient in the *cact* homolog, I- $\kappa$ B $\alpha$ , while other genes require additional activation pathways (Beg et al., 1995).

We are aware that the existence of two distinct regulatory pathways controlling the expression of antimicrobial genes raises the question of the possible existence of distinct recognition mechanisms. To date, our information on non-self-recognition in insect immunity is fragmentary, and this area is now becoming a major field of interest (discussed by Hultmark, 1993; Hoffmann, 1995).

### Role of the Antimicrobial Peptides in the Resistance to Infection in *Drosophila*

The presence of antimicrobial peptides has been reported over the last 15 years in as disparate organisms as plants, arthropods, mollusks, amphibians, and mammals. Some of these molecules share sequence similarities bridging the phylogenetic distances (e.g., brevinins, cecropins, and defensins) while others appear specific to a given class of organisms (reviewed by Hetru et al., 1994; Boman et al., 1995; Broekaert et al., 1995). The antimicrobial peptides have been proposed to play a role in limiting bacterial or fungal proliferation and thus in contributing to the host defense. The generation of the *imd*; *TI* double mutants, in which the synthesis of all antimicrobial peptides is impaired, has provided a model to investigate the *in vivo* relevance of the immune-induced synthesis of antimicrobial peptides in the host defense. Our results clearly demonstrate that the *imd* and *TI* pathways are both essential for full antimicrobial resistance in *Drosophila*. Our study also establishes a correlation between the impairment of antifungal gene induction and reduced resistance to fungal infection and, conversely, between the impairment of antibacterial gene induction and reduced resistance to bacterial infection. Although we cannot exclude the possibility that these mutations also affect other immune mechanisms (proteolytic cascades, cellular reactions essential for the host defense), our results show that under our experimental conditions the insects could not survive an infection when antimicrobial gene induction was impaired, most likely, as illustrated for *E. coli*, because they were unable to control the proliferation of the microorganisms.

### Concluding Remarks

Several studies have underlined the parallels between the cytokine-induced activation of NF- $\kappa$ B and the *TI*-mediated activation of *dl*. It has also been proposed that these parallels extend to the *Drosophila* immune response. By providing a demonstration that the components of the dorsoventral pathway play an essential role in the potent antimicrobial response of *Drosophila*, we confirm that this activation pathway is indeed an ancient regulatory cascade involved in host defense. The recent identification of the N-protein, which plays a role in the resistance of tobacco plants to tobacco mosaic virus, has highlighted sequence similarities between the N-terminal domain of this protein and the cytoplasmic domain of both the IL-1 and *TI* receptors (Whitham et al., 1994). By showing that *TI* is also involved in the immune response, we lend further credit to the idea that the host defense in higher eukaryotes involves a common regulatory pathway, mediated by a homologous protein domain present in these proteins (Dinesh-Kumar et al., 1995).

The powerful genetic system of *Drosophila* provides an excellent model for further dissection of the control mechanisms of primordial immunity.

### Experimental Procedures

#### *Drosophila* Stocks

The lines used in this study have been described elsewhere (Anderson and Nüsslein-Volhard, 1984, 1986; Lemaître et al., 1995a, 1995b).

Mutants in *pll*, *tub*, and especially *TI* have been reported to exhibit some lethality during the larval stage and larvae and pupae of abnormal size (Letsou et al., 1991; Gertulla et al., 1988; Hecht and Anderson, 1993). To obtain *TI* larvae and adults, we have used two temperature-sensitive alleles of *TI* (*TI<sup>ts2</sup>* and *TI<sup>ts4</sup>*) that exhibit a strong phenotype only when raised at 29°C (Gertulla et al., 1988). *TI*-deficient mutants were reared at 18°C and shifted at the adult stage to 29°C. Since strong alleles of *cact* result in larval/pupal lethality (Roth et al., 1991), we have used here the strongest viable allele of *cact* (*cact<sup>42</sup>*). All experiments were performed at 25°C except when otherwise stated. For complete descriptions of the marker genes and balancer chromosomes, see Lindsley and Zimm (1992).

### Injury and Survival Experiments

Bacterial challenge was performed by pricking adults with a thin needle previously dipped into a concentrated bacterial culture of *E. coli* and *Micrococcus luteus*.

Survival experiments were carried out in the same conditions for each genotype tested. Groups of 20 adults, aged 2–4 days, were challenged and transferred to a fresh vial every 4 days. Flies that died within 3 hr (less than 5% of the total) after challenge were not considered in the analysis. In preliminary experiments, we observed that survival rates could strongly depend on the genetic background. For example, we noted that homozygous *ebony* (*e*) flies exhibited a low viability after challenge as previously reported by Flyg and Boman (1988). To test the survival of the mutation under analysis, we used strains with mutated chromosomes carrying a minimum of markers and exhibiting a good viability.

### RNA Preparation and Analysis

Crosses were performed at 25°C, and 2- to 4-day-old adult flies were collected. Total RNA extraction and Northern blotting experiments were performed as in Lemaître et al. (1995a). The following probes were used: attacin cDNA (Asling et al., 1995), cecropin A1 cDNA (Kylsten et al., 1990), defensin cDNA (Dimarq et al., 1994), dipterin cDNA (Wicker et al., 1990), drosocin cDNA (Bulet et al., 1993), drosomycin cDNA (Fehlbaum et al., 1994), rp49 cDNA (a PCR fragment of approximately 400 bp generated between two oligonucleotides designed after the rp49 coding sequence (O'Connell and Rosbach, 1984). The cecropin A1 probe cross-reacts with cecropin A2 transcripts (Kylsten et al., 1990). We observed that chromosomal markers associated with the mutations that were tested (*b*, *pr*, *cn*, *bw*, *sp*, *ru*, *st*, *e*, *ca*, *cu*, *kar*, *red*, *sbd*, *th*, *ri*, *roe*, *p*, *h*, *sr*, *mwh*) did not alter the induction of the genes encoding drosomycin, cecropin A and dipterin. Poly(A) RNA extraction was prepared as in Lemaître et al. (1995b), except that extraction of total RNA was performed with the RNA Trizol (GIBCO BRL) method.

### Acknowledgments

Correspondence should be addressed to J. A. H. The authors are indebted to Dr. Christiane Nüsslein-Volhard, Dr. Kathryn Anderson, and Dr. Steve Wasserman for sending flies carrying dorsoventral mutations or plasmids encoding dorsoventral genes, to Dr. Kathy Matthews for sending current stocks of the Bloomington Center, and to Dr. Dan Hultmark and Dr. Mitchel Dushay for the gift of attacin and cecropin A1 cDNAs. We thank our colleagues Pascale Fehlbaum and Dr. Philippe Bulet, who performed the high pressure liquid chromatography purification of the drosomycin peptide, and Dr. René Lanot, Dr. Marie Meister, Dr. Jean Luc Imler, Dr. Sarah Ades, and Dr. Dominique Ferrandon for stimulating discussions. The technical assistance of Reine Klock and Raymonde Syllas is gratefully acknowledged. This work was supported by the Centre National de la Recherche Scientifique, the University Louis Pasteur of Strasbourg, Rhone Poulenc, and the Human Frontiers Science Programme.

Received March 13, 1996; revised August 8, 1996.

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# Analogy Between *Drosophila* and Mammalian TRAF Pathways

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**Abstract.** A central event in innate immunity is the activation of the NF- $\kappa$ B signaling pathway and up-regulation of NF- $\kappa$ B-dependent defense genes. Attack of mammals as well as of insects by microorganisms leads, among other things, to the activation of receptors of the Toll-like receptor group. Various adaptor proteins involving members of the TNF receptor-associated factor (TRAF) family channel these receptor-generated signals to conserved intracellular kinase cascades that finally lead to the activation of NF- $\kappa$ B and JNK. In vertebrates, TRAF proteins link these pathways also to IL-1R-related molecules and members of the TNF receptor superfamily, which orchestrate a variety of immunoregulatory processes of the innate but also of the adaptive immune system. In this review, we will focus on the similarities but also the differences in TRAF-dependent signaling pathways of mammals and insects.

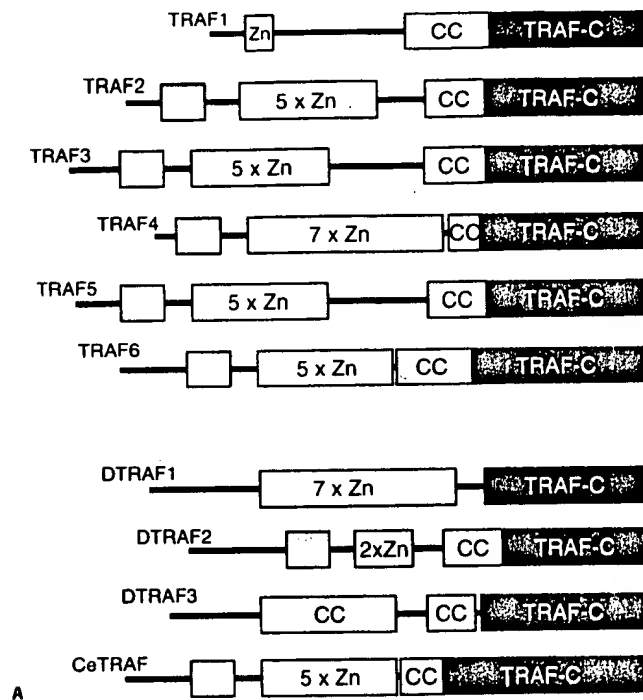
## 1

### Introduction

The "TNF receptor-associated factor" (TRAF) family comprises a group of adaptor/scaffold proteins involved in the activation and regulation of the NF- $\kappa$ B pathway or mitogen-activated protein kinase signaling cascades. Currently, six TRAF proteins, designated as TRAF1 to TRAF6, have been described in mammals (Wajant et al. 2001; Chung et al. 2002), three (DTRAF1, DTRAF2 and DTRAF3) in flies (Liu et al. 1999; Grech et al. 2000) and one in *Caenorhabditis elegans* (Wajant et al. 1998) (Fig. 1A). The TRAF proteins are characterized by a carboxy-terminal homology domain, the TRAF domain, which is required for self-association of these molecules. In addition, the TRAF domain enables these proteins to undergo a huge variety of protein - protein interactions. The TRAF domain can be subdivided into the highly conserved TRAF-C subdomain, consisting of an eight-stranded anti-parallel  $\beta$ -sandwich structure and a less conserved amino-terminal part, the TRAF-N domain, which is organized as a coiled-coil (Wajant et al. 2001; Chung et al. 2002). The TRAF domains form trimers reminiscent in their structure to a trefoil, with the three TRAF-C domains as leaves and the trimerized TRAF-N domains as the stalk (Park et al. 1999; McWhirter et al. 1999; Ye et al. 1999; Ni et al. 2000). With the excep-

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**Fig. 1.** The TRAF protein family. **A** Domain architecture of TRAF proteins. Note that the carboxy-terminal TRAF-C domain and the foregoing coiled-coil domain (TRAF-N domain) together form the TRAF domain. *R* indicates a RING domain and *Zn* refers to the zinc finger motif. **B** Protein-protein interaction capabilities of a generalized TRAF protein. For details of the plethora of TRAF-interacting proteins, see Wajant et al. (2001)

tion of DTRAF1, DTRAF3 and TRAF1, the amino-terminal parts of all TRAF proteins contain a RING domain followed by a regularly spaced cluster of five or seven zinc fingers (Fig. 1A). While DTRAF1 lacks the RING domain but has also seven zinc fingers, the amino-terminal part of TRAF1 contains a single zinc finger but no additional homologies to the other TRAF proteins. The amino-terminal part of DTRAF3 is also unique among the TRAF proteins and contains an extended coiled-coil region (Grech et al. 2000). As the TRAF domain of TRAF1 is highly homologous to the TRAF domain of TRAF2, the atypical RING/zinc domain-less architecture of the TRAF1 amino-terminal domain most likely arose late in evolution (Zapata et al. 2000; Wajant et al. 2001). There is overwhelming evidence that the TRAF domain of TRAF proteins acts as an assembly platform that interacts with members of the TNF

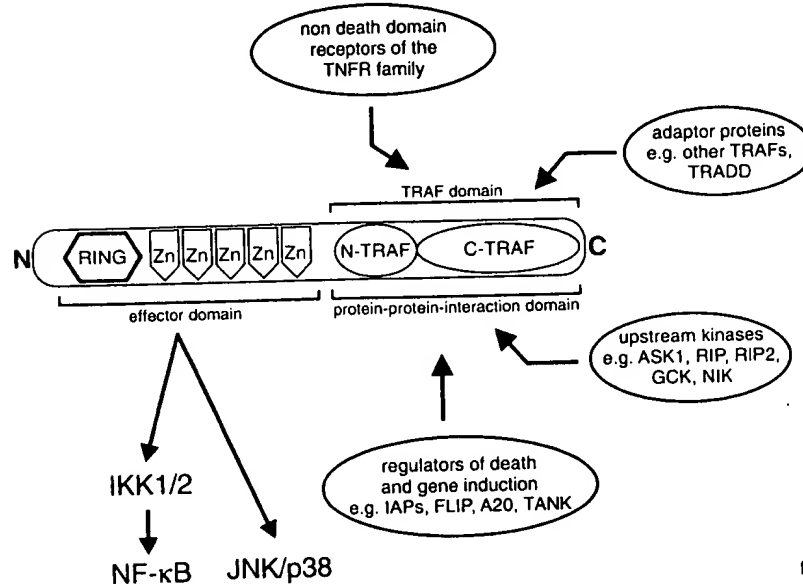


Fig. 1. Continued

receptor and IL-1R/Toll receptor families, additional adaptor proteins and a variety of kinases as well as modulatory proteins regulating these kinases (Wajant et al. 2001; Chung et al. 2002; Fig. 1B). In contrast, the role of the amino-terminal RING/zinc domains of TRAF proteins is less understood. It has been recently shown that TRAF6 can act as an E3 ligase most likely catalyzing its own ubiquitination with atypical lysine-63-linked polyubiquitin chains (Deng et al. 2000; Wang et al. 2001). Moreover, ubiquitinated TRAF6 has been implicated in the activation of TGF- $\beta$ -activated kinase (Tak1), a mitogen-activated protein kinase kinase kinase (MAP3K) involved in the stimulation of the NF- $\kappa$ B-inducing I- $\kappa$ B kinase (IKK) complex (Wang et al. 2001). In addition, for TRAF2 and TRAF6, interactions of their RING/zinc domains with MEKK1 (Baud et al. 1999), another MAP3K, and IKK2 (Devin et al. 2001), a subunit of the IKK complex, have been found. Overexpression of TRAF proteins leads to the activation of TRAF-associated signaling pathways. It is noteworthy that, while the expression of the sole RING/zinc domains is not sufficient to trigger the activation of TRAF-associated signaling pathways, the artificial oligomerization of these domains restores signaling capacities attributed to the corresponding complete TRAF proteins (Baud et al. 1999; Wang et al. 2001). Taken together, these findings suggest that TRAF proteins not only act as scaffolds to

bring together receptors and cytosolic signaling intermediates, but may also have a role in the activation of the recruited effector molecules. In recent years, additional proteins have been identified that contain domains with significant homologies to the TRAF domain of the "classical" TRAF proteins, the so-called TEFs (TRAF domain-encompassing factors) (Zapata et al. 2001). However, the role of these TEFs and their relationship to TRAF-associated functions are still completely unclear.

## 2

### TRAF-Dependent Signaling Pathways in Mammalian and Insect Innate Immunity

There is a common need of vertebrates and invertebrates to protect themselves against the attack of a wide variety of pathogens. Both mammals and insects have developed a range of mechanisms enabling them to respond rapidly against potentially harmful microorganisms. This innate immunity response is essentially based on the sensing of microorganism-derived substances by germ-line encoded pattern recognition proteins and induction of defense mechanisms, in particular activation of phagocytic cells and transcriptional up-regulation of anti-microbial peptides. In insects, anti-microbial peptides are either induced in epithelial cells to act locally or are produced in the fat body, the insect analogue of the vertebrate liver, to act systemically (Meister et al. 2000). In mammals, anti-microbial peptides are predominantly expressed in epithelial tissue and comprise constitutively expressed as well as inducible forms (Yang et al. 2001; Lehrer and Ganz 2002). In vertebrates, innate immunity is additionally tightly linked to the adaptive immune system that responds to pathogens with the selective expansion of antigen-specific T and B cells, which are selected from a huge variety of clones generated by somatic chromosomal DNA rearrangement in the T- and B-cell receptor genes. In *Drosophila*, seven anti-microbial peptides exist: drosomycin, which is predominantly active against fungi, metchnikowin, which exerts anti-fungal and anti-bacterial activity, and attacin, cecropin, defensin, dipterecin and drosocin, which mainly act against bacteria (Meister et al. 2000). Infection-induced transcriptional up-regulation of these anti-microbial peptides is achieved by two distinct signaling pathways: the immune deficiency (Imd)-related pathway (Lemaitre et al. 1995) and the Toll pathway (Lemaitre et al. 1996). Both pathways lead to the activation of members of the NF- $\kappa$ B/Rel transcription factor family (see Appendix 1) but are well separated upstream of these factors. Thus, mutations affecting/inhibiting a component of one pathway do not interfere with the functionality of the other pathway (Table 1). While the Toll pathway predominantly induces drosomycin and the Imd pathway up-regulates selectively dipterecin and drosocin, the remaining anti-microbial peptides require both pathways for maximal induction (Lemaitre et al. 1996; Levashina et al.

1998). Distinct categories of microbes differentially stimulate the Toll and Imd pathways. The Toll pathway is triggered by fungi or Gram-positive bacteria, whereas the Imd pathway is activated by Gram-negative bacteria (Silverman and Maniatis 2001; Hoffmann and Reichhart 2002). Microbial infection also leads to activation of the c-Jun-N-terminal kinase (JNK) (see Appendix 2) and JAK/STAT pathways (Sluss et al. 1996; Lagueux et al. 2000; Levashina et al. 2001). While the latter induces up-regulation of complement-related proteins implicated in the opsonization of Gram-negative bacteria, the importance of the JNK pathway for the *Drosophila* innate immune response is still unclear.

With regard to intracellular signaling intermediates, the Imd and Toll pathways in *Drosophila* are non-redundant despite utilizing in principle related pathways. Remarkably, phylogenetically conserved components of both the Imd and the Toll pathways work in combination in the mammalian Toll-like receptors/IL-1R pathway (see below). Noteworthy, the Imd pathway utilizes in addition several conserved proteins, which have been implicated in apoptosis-induction in mammals and *Drosophila*.

## 2.1

### The Toll Pathway in Innate Immunity of *Drosophila*

The Toll pathway has been originally identified due to its pivotal role in the dorsoventral patterning of the *Drosophila* embryo (Belvin and Anderson 1996). Toll is a transmembrane receptor and the name-giving part of a signaling pathway that transduces extracellular "information" to the nucleus to initiate a transcriptional response towards these extracellular stimuli (Hashimoto et al. 1988). Toll consists of an extracellular domain comprising several leucine-rich repeats, a single transmembrane domain and an intracellular part containing a conserved domain of about 150 amino acids. The conserved intracellular domain is also found in other Toll-like receptors (TLRs), in the IL-1R family as well as in the MyD88 adaptor proteins and is therefore named the Toll/IL-1R homology (TIR) domain (Silverman and Maniatis 2001). In dorsoventral patterning of the *Drosophila* embryo, Toll gets activated by a cleavage product of the cysteine knot growth factor-like protein Spätzle, which consists of a disulfide-linked dimer of the carboxy-terminal 106 amino acids of the complete Spätzle protein (Morisato and Anderson 1994; DeLotto and DeLotto 1998). Proteolytic activation of Spätzle in dorsoventral patterning is achieved by a serine protease cascade comprising Gastrulation Defective, Snake, and Easter (LeMosy et al. 1999). Proteolytic activation of Spätzle has also an essential role in activation of Toll in innate immunity, but in this case this process is independent from the aforementioned protease cascade (Lemaitre et al. 1996; Levashina et al. 1999). Thus, loss-of-function mutants of Gastrulation Defective, Snake, and Easter have no inhibitory effect on Toll-mediated up-regulation of drosomycin during infection with Gram-positive bacteria or fungi, whereas a loss-of-function mutant of Spätzle strongly reduces these



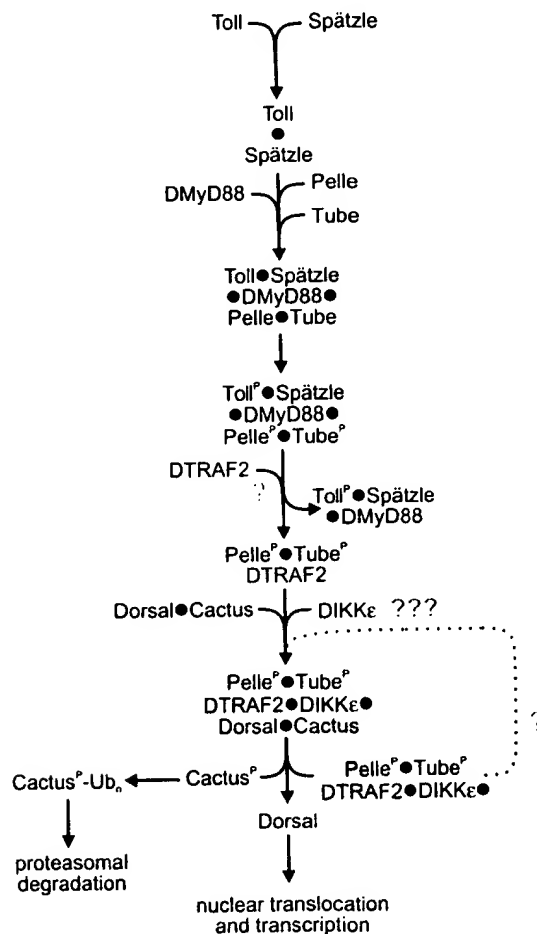
Table 1. Innate immunity in mutant *Drosophila* strains

Gene	Type of mutation	Inducible activity		Constitutive activity		References
		Toll	Imd	Toll	Imd	
Wild type						
semmelweis (PGRP-SA)	Loss-of-function	Yes	Yes	No	No	Michel et al. (2001)
semmelweis (PGRP-SA)	Loss-of-function in toll	No (Gram-positive)	Yes	No	No	
semmelweis (PGRP-SA)	Loss-of-function in toll gain-of-function flies	Yes (fungi)	n.d.	Yes	No	Michel et al. (2001)
semmelweis (PGRP-SA)	Loss-of-function in necrotic loss-of-function flies	n.d.	n.d.	Yes	No	Michel et al. (2001)
necrotic (Spn43ac)	Loss-of-function	Yes	Yes	Yes	No	Levashina et al. (1999)
necrotic (Spn43ac)	Loss-of-function in toll or spätzle or snake or gastrulation defective loss-of-function flies	No	Yes	No	No	Levashina et al. (1999)
easter	Loss-of-function	Yes	Yes	No	No	Lemaitre et al. (1996)
snake	Loss-of-function	Yes	Yes	No	No	Lemaitre et al. (1996)
spätzle	Loss-of-function	No	Yes	No	No	Lemaitre et al. (1996)
toll	Loss-of-function	No	Yes	No	No	Lemaitre et al. (1996)
dmyd88	Gain-of-function	Yes	Yes	Yes	No	Lemaitre et al. (1996)
dmyd88	Overexpression in wild-type flies			Yes	No	Tauszig-Delamasure et al. (2002)
dmyd88	Overexpression in pelle loss-of-function flies			Yes	No	Tauszig-Delamasure et al. (2002)
dmyd88	Overexpression in tube loss-of-function flies			No	No	Tauszig-Delamasure et al. (2002)
pelle	Loss-of-function	No	Yes	No	No	Lemaitre et al. (1996)
tube	Loss-of-function	No	Yes	No	No	Lemaitre et al. (1996)
cactus	Loss-of-function	Yes	Yes	Yes	No	Lemaitre et al. (1996)
dif	Loss-of-function	Strongly reduced	Yes	No	No	Rutschmann et al. (2000a)
dif	Loss-of-function in cactus loss-of-function flies	n.d.	n.d.	No	n.d.	Rutschmann et al. (2000a)

ird7 (PGRP-LC)	Loss-of-function	Normal	Strongly reduced	No	Choe et al. (2002); Ramet et al. (2002)
imd	Overexpression in imd loss-of-function flies	Yes	Yes	No	Georgel et al. (2001)
imd	Overexpression in kenny loss-of-function	Yes	No	No	Georgel et al. (2001)
imd	Overexpression in dredd loss-of-function	Yes	No	No	Georgel et al. (2001)
dtak1	Loss-of-function	Yes	No	No	Vidal et al. (2001)
dtak1	Heat shock-induced in wild-type flies	Yes	No	No	Vidal et al. (2001)
dtak1	Heat shock-induced in ird5			No	Vidal et al. (2001)
dtak1	Loss-of-function flies			No	Vidal et al. (2001)
dtak1	Heat shock-induced in kenny loss-of-function flies			No	Vidal et al. (2001)
dtak1	Heat shock-induced in imd loss-of-function flies			No	Vidal et al. (2001)
kenny	Loss-of-function	Yes	No	No	Rutschmann et al. (2000a)
ird5	Loss-of-function	Yes	No	No	Lu et al. (2001)
dredd	Loss-of-function	Yes	No	No	Leulier et al. (2000)
dredd	Heat shock-induced in wild type flies			No	Vidal et al. (2001)
dredd	Heat shock-induced in kenny loss-of-function flies			No	Vidal et al. (2001)
dredd	Heat shock-induced in imd loss-of-function flies			No	Vidal et al. (2001)
relish	Loss-of-function	Yes	No	No	Hedengren et al. (1999)
toll + imd	Both loss-of-function	No	No	No	Lemaitre et al. (1996); Levashina et al. (1998)

responses (Lemaitre et al. 1996). In agreement with a distinct proteolytic cascade controlling Spätzle activation in Toll-dependent signaling in innate immunity, Spn43Ac, a serine protease inhibitor of the serpin family, prevents constitutive processing of Spätzle, but has no effect on dorsoventral patterning or the Imd pathway (Levashina et al. 1999). Recognition of Gram-positive bacteria and fungi and activation of Toll diverges upstream of Spätzle processing. This became evident from the identification of a soluble peptidoglycan recognition protein (PGRP-SA) that is essentially involved in Toll activation by several Gram-positive bacteria, but has no role in the anti-fungal response (Michel et al. 2001). How recognition of Gram-positive bacteria by PGRP-SA results in the processing of Spätzle remains to be elucidated. The Spätzle processing-dependent mode of Toll activation in innate immunity of *Drosophila* suggests that Toll itself does not directly recognize microbe-derived molecular patterns. This is in striking contrast to innate immunity in mammals where free or protein complexes of microbial products directly stimulate TLRs without the need of activated proteases (Akira et al. 2001). Moreover, in mammals, all TLRs seem to be involved in innate immunity, whereas in *Drosophila*, there is yet no evidence for an anti-microbial function of most of the Toll-related receptors (Tauszig et al. 2000).

Activation of Toll results in the recruitment of the *Drosophila* homologue of the mammalian adapter protein "myeloid differentiation primary response gene 88" (DmMyD88), the serine/threonine kinase Pelle and Tube. Loss-of-function mutants of these proteins interfere with induction of the Toll pathway-specific peptide drosomycin upon fungal or Gram-positive bacteria infection but have no impact on the Imd pathway-mediated response (Lemaitre et al. 1996; Tauszig-Delamasure et al. 2002). Like its mammalian counterpart, DmMyD88 consists of an amino-terminal death domain followed by a TIR domain and a phylogenetically non-conserved carboxy-terminal extension. DmMyD88 binds Toll by homophilic interaction of the TIR domains of both proteins (Horng and Medzhitov 2001; Tauszig-Delamasure et al. 2002). DmMyD88 is also able to bind Pelle (Horng and Medzhitov 2001; Tauszig-Delamasure et al. 2002), which itself interacts directly with Toll in its non-phosphorylated form by its carboxy-terminal catalytic domain (Shen and Manley 1998). In addition, Pelle interacts with the amino-terminal death domain of Tube by its own amino-terminal death domain (Grosshans et al. 1994; Galindo et al. 1995). Again, this interaction is predominantly observed with non-phosphorylated Pelle (Edwards et al. 1997). Pelle phosphorylates Toll, Tube and itself in vitro (Grosshans et al. 1994; Shen and Manley 1998). Expression of a kinase-inactive mutant of Pelle results in increased localization of Tube to sites of Toll receptor signaling in *Drosophila* embryos, suggesting that Pelle-mediated phosphorylation events trigger the release of one or more components from the Toll signaling complex (Towb et al. 2001). Up-regulation of drosomycin by overexpression of DmMyD88 is abrogated in flies with loss-of-function mutants of Tube or Pelle, placing DmMyD88 upstream of these molecules in the Toll pathway (Tauszig-Delamasure et al. 2002; Fig. 2).

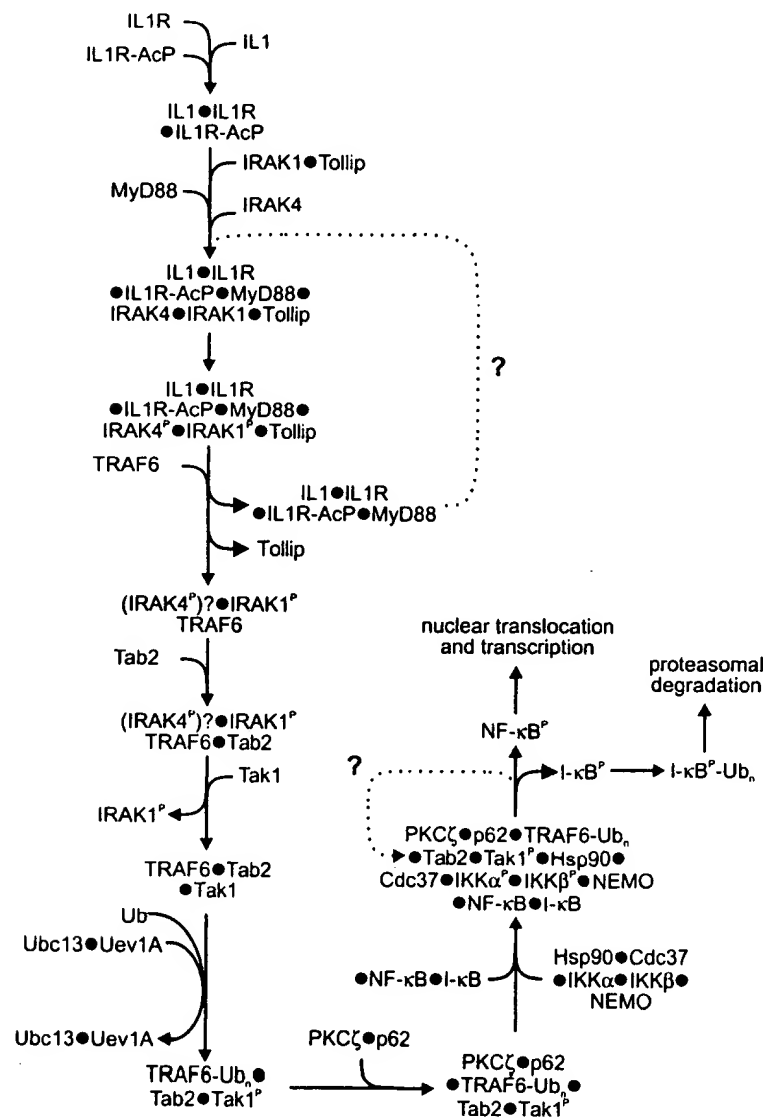


**Fig. 2.** Model of Spätzle-mediated activation of Dorsal. Events in the pathway currently not directly substantiated by experimental evidence are labeled with “?”

Signal initiation by activated IL-1R or mammalian TLRs is very similar to the mechanisms described above for activated Toll. Again, the adaptor protein MyD88 is recruited to the activated receptor by homophilic TIR domain interaction (Muzio et al. 1997; Wesche et al. 1997; Burns et al. 1998; Medzhitov et al. 1998). Analysis of MyD88 knockout mice revealed an essential role of this adaptor protein for signal transduction by the receptors of IL-1 and IL-18 as

well as for some of the TLRs (Adachi et al. 1998; Kawai et al. 1999; Takeuchi et al. 2000). Moreover, there is evidence that the MyD88-independent TLRs utilize the MyD88-related protein MAL (MyD88-adaptor-like) in a similar way (Fitzgerald et al. 2001). In addition, the functions of Pelle and Tube in Toll signaling are at least partly mirrored by the mammalian homologues of Pelle, the members of the "IL-1 receptor-associated kinase" (IRAK) family. In humans, four IRAKs have been identified: IRAK-1 (Cao et al. 1996), IRAK-2 (Muzio et al. 1997), IRAK-M (Wesche et al. 1999) and IRAK-4 (Li et al. 2002). The IRAKs are recruited to the IL-1R in a ligand-dependent manner and, like Pelle, interact with the concomitantly recruited MyD88 protein (Cao et al. 1996; Muzio et al. 1997; Wesche et al. 1999; Li et al. 2002; Fig. 3). Reconstitution experiments in IRAK-1-deficient cells, where IL-1-induced activation of NF- $\kappa$ B and JNK is strongly reduced, revealed that IRAK-M and IRAK-2 are able to replace IRAK-1 in IL-1 signaling (Li et al. 1999; Wesche et al. 1999). These studies further revealed that the kinase activity of these enzymes is dispensable for their role in IL-1 signaling. Nevertheless, kinase-dead mutants of IRAK-1, IRAK-2 or IRAK-M are phosphorylated and this is tightly correlated with the capability of these mutants to induce NF- $\kappa$ B (Li et al. 1999). Like its *Drosophila* counterpart, MyD88 acts downstream of the receptor but upstream of IRAK-4 and IRAK-1 (Suzuki et al. 2002). It has been recently shown that IRAK-4 phosphorylates IRAK-1 in response to IL-1 (Li et al. 2002). Moreover, the kinase activity of IRAK-4 is indispensable for its NF- $\kappa$ B-inducing capacity (Li et al. 2002). In agreement with the functional redundancy of IRAK-1, IRAK-2 and IRAK-M and the unique role of IRAK-4 in IL-1-mediated NF- $\kappa$ B activation, mice deficient in IRAK-1 show residual IL-1-induced NF- $\kappa$ B activity (Kanakaraj et al. 1998) whereas mice lacking IRAK-4 are completely blocked in this response (Suzuki et al. 2002). So far investigated, IRAK-1 and IRAK-4 have a similar function in the signal transduction by some of the human TLRs and the IL-18R (Kanakaraj et al. 1998; Suzuki et al. 2002). Taken together, there is a high degree of similarity in the molecular mode of function between MyD88 and IRAK-4 in mammalian cells and DmMyD88 and Pelle in *Drosophila*. Moreover, with respect to their interplay with MyD88/DmMyD88 and IRAK-4/Pelle, the phylogenetically unrelated proteins IRAK-1 and Tube also act in a comparable manner. Phosphorylation of IRAK-1 in the receptor complex leads to the interaction with the adaptor protein TRAF6 and the dissociation of both molecules from the IL-1R complex (Qian et al. 2001). Remarkably, there is evidence that Pelle is involved in a similar way in the release of Tube from the Toll complex (Towb et al. 2001). Phosphorylation of IRAK-1 may also result in its ubiquitination and proteasomal degradation (Yamin and Miller 1997), but these events are apparently dispensable for its NF- $\kappa$ B-inducing function (Qian et al. 2001).

Three members of the TRAF protein family have been recently identified in *Drosophila* (DTRAF1, DTRAF2, DTRAF3), but the potential involvement of these proteins in the Toll pathway is poorly understood. In agreement with a role of DTRAF2 in Toll signaling, overexpression of this TRAF protein was suf-



**Fig. 3.** Model of IL-1-induced activation of NF-κB. Events in the pathway currently not directly substantiated by experimental evidence are labeled with “?”



ing to a role of JNK in the Imd pathway too (Sluss et al. 1996). Similarly, bifurcation of the *Drosophila* JNK and NF- $\kappa$ B signaling pathways at the level of Pelle may also occur in mammalian TLR signaling as a dominant-negative mutant of TRAF6 interferes with NF- $\kappa$ B activation by TLRs, but failed to block activation of JNK (Muzio et al. 1998). However, at least in IL-1R and IL-17R signaling the situation seems to be different, because in TRAF6-deficient mice, both NF- $\kappa$ B and JNK activations are impaired in response to IL-1 and IL-17 (Lomaga et al. 1999; Schwandner et al. 2000). The TRAF6 knockout mice have also a defect in LPS/TLR-induced NF- $\kappa$ B activation, but unfortunately TLR-induced JNK activation was not investigated in these mice yet. Nevertheless, even in the case of IL-1R and IL-17R signaling, IRAK-1 seems to be the bifurcation point of NF- $\kappa$ B and JNK activation as different domains of the molecule are required to mediate these responses (Li et al. 2001).

In the IL-1-induced NF- $\kappa$ B pathway, TRAF6 most likely has multiple, yet poorly understood functions. TRAF6 acts as an E3 ligase which catalyses, together with a dimeric ubiquitin-conjugating enzyme complex consisting of Ubc13 and Uev1A, its own ubiquitination (Deng et al. 2000; Wang et al. 2001). Ubiquitinated TRAF6 then facilitates Tak1-dependent activation of the IKK complex (Wang et al. 2001). Remarkably, polyubiquitin chains are unusually linked to TRAF6 through lysine-63 (K63), instead of lysine-48 (K48) (Deng et al. 2000; Wang et al. 2001). While ubiquitination of TRAF6 is necessary for Tak1-dependent activation of the IKK complex, proteasomal degradation, which normally occurs with K48 - ubiquitin linked proteins, is dispensable (Deng et al. 2000). Noteworthy, IFN- $\gamma$  induces rapid proteasomal degradation of TRAF6 in osteoclasts, opening the possibility that TRAF6 is also the target of K48 ubiquitination (Takayanagi et al. 2000). Nevertheless, how Tak1 is activated by ubiquitinated TRAF6 is still unknown. DTRAF2, and deletion mutants of DTRAF2 containing the RING domain, are modified upon transient expression in *Drosophila* SL2 cells (Shen et al. 2001). As this modification has been ruled out to be phosphorylation but leads to a ladder-like staining in western blotting experiments (Shen et al. 2001), most likely DTRAF2 is also ubiquitinated during Toll signaling. However, a role of DTRAF2 modification/ubiquitination for Dorsal activation or DTRAF2 degradation remains to be established. TRAF6 also acts, together with p62, as an adaptor joining the IKK complex and the atypical protein kinase C zeta (PKC $\zeta$ ), a kinase involved in transactivation of p65 and/or tissue-specific activation of IKKs (Sanz et al. 2000; Leitges et al. 2001). A similar p62-like adaptor function has also been proposed for the phylogenetically conserved ECSIT (evolutionary conserved signaling intermediate in Toll pathway) protein, which concomitantly interacts with MEKK1 and TRAF6/DTRAF2 (Kopp et al. 1999). In *Drosophila*, a homologue of Tak1 (DTak1) exists, but surprisingly, the DTak1 protein is essentially involved in the Imd pathway and dispensable for Toll-induced activation of NF- $\kappa$ B (Vidal et al. 2001). In IL-1 signaling, TRAF6, which is predominantly localized at the plasma membrane (Qian et al. 2001), is not the only protein that translocates into the cytosol in complex with phosphorylated IRAK-1. Like-

efficient to drive a Dorsal-regulated reporter gene (Shen et al. 2001). Moreover, DTRAF2 and Pelle synergistically activate Dorsal and a weak interaction of both molecules can be observed in vitro (Shen et al. 2001). Deletion mutant analyses of Pelle showed that it interacts with DTRAF2 via its kinase domain (Shen et al. 2001). Interestingly, the RING/zinc finger domain of DTRAF2 alone interacts much stronger with Pelle as compared to the complete protein (Shen et al. 2001). Moreover, the RING/zinc domain of DTRAF2 is more potent than complete DTRAF2 in the activation of a Dorsal-driven reporter gene, and synergizes, in this regard, with Pelle too. An amino-terminal truncated deletion mutant of DTRAF2, solely comprising the TRAF domain of the protein, is nevertheless able to activate Dorsal (Shen et al. 2001). This is very surprising because in mammalian cells, the corresponding TRAF6 deletion mutant, but also similar mutants of other TRAF proteins, displayed a dominant-negative effect on the respective signaling pathways. Thus, the interaction of TRAF6 and IRAK-1 or IRAK-2 relies on the TRAF domain of TRAF6 (Muzio et al. 1997) and the amino-terminal regulatory domain of IRAK-1 and IRAK-2 (Li et al. 2001), whereas the interaction of DTRAF2 and Pelle seems to be based on the RING/zinc finger domain of DTRAF2 and the kinase domain of Pelle (Shen et al. 2001). Therefore, at a first glance, the molecular mode of TRAF – kinase interaction seems not to be conserved between mammals and flies. However, for the reasons outlined above, IRAK-4 may be the only kinase of the IRAK family that fulfills a Pelle analogue role in mammals, whereas the other IRAK family members functionally behave like *Drosophila* Tube. Thus, it remains to be clarified (1) whether there is an interaction between Tube and DTRAF2, qualitatively reflecting the IRAK-1 interaction with TRAF6, and (2) whether there is an interaction between the kinase domain of IRAK-4 and the RING/zinc finger domain of TRAF6.

Although mammalian TRAF4 seems not to be involved in NF- $\kappa$ B signaling, its *Drosophila* counterpart DTRAF1 has been implicated in Toll signaling as it interacts via its TRAF domain with the death domain-containing regulatory domain of Pelle (Zapata et al. 2000). Thus, DTRAF1 and DTRAF2 utilize different domains (TRAF domain versus RING/zinc finger domain) to interact with different parts of Pelle (regulatory domain versus kinase domain). Although an amino-terminal deletion mutant of DTRAF1 interferes with Pelle-induced NF- $\kappa$ B activation upon overexpression in mammalian cells, micro-injection of the respective mRNA in *Drosophila* had no effect on dorsoventral patterning (Zapata et al. 2000), arguing against a major role of DTRAF1 in Toll-induced activation of NF- $\kappa$ B/Dorsal. DTRAF1 was originally identified due to its interaction with Misshapen, a mitogen-activated protein kinase kinase kinase (MAP4K) that is involved in *Drosophila* in the activation of the JNK mitogen-activated protein kinase module (Liu et al. 1999). It is therefore tempting to speculate that DTRAF1 is involved in Toll-mediated activation of the JNK pathway. Indeed, ectopic expression of Tube and Pelle activates the JNK pathway in mammalian cells (Bacher et al. 2001). Moreover, it has been shown that the *Drosophila* homologue of JNK can be activated by LPS, point-

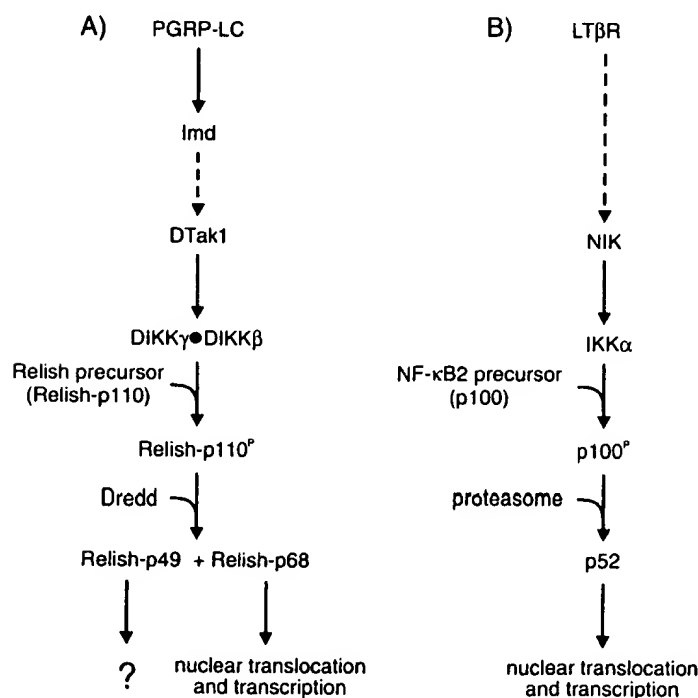
wise, Tab2, a primarily membrane-associated protein acting as an adaptor for TRAF6 interaction with Tab1 and Tak1, behaves in a similar way (Takaesu et al. 2001). Thus, IL-1-induced phosphorylation of IRAK-1 results in the appearance of a cytosolic complex containing phosphorylated IRAK-1, TRAF6, Tak1 and Tab2, the latter being involved in the activation of Tak1. It is currently unknown whether D'Tak1 is dependent in its function on Tab1/2-related factors.

TRAF6-mediated activation of Tak1 leads to the activation of the kinases of the IKK complex. The activated IKK complex phosphorylates I- $\kappa$ B on conserved serine residues thereby marking this protein for ubiquitination and subsequent proteasomal degradation (see Appendix 1). In non-stimulated cells, I- $\kappa$ B is complexed with dimers of the Rel/NF- $\kappa$ B family of transcription factors (see Appendix 1). Thus, as a consequence of I- $\kappa$ B degradation, NF- $\kappa$ B transcription factors are released to translocate to the nucleus. Likewise, activation of the Toll pathway in *Drosophila*, results in the phosphorylation of the regulatory domain of the *Drosophila* I- $\kappa$ B homologue Cactus and its subsequent proteasomal degradation (Spencer et al. 1999). The microbial activation of the Toll pathway in larvae causes Cactus degradation, and results in nuclear translocation of Dorsal and Dif, two Rel/NF- $\kappa$ B proteins, whereas in adult flies only Dif is required for the anti-fungal response (Manfrulli et al. 1999; Meng et al. 1999; Rutschmann et al. 2000a). The Cactus kinase, which would close the gap between Pelle-DTRAF2 interaction and Cactus phosphorylation, has not been identified yet. DLAK/DmIKK $\beta$  (Kim et al. 2000; Silverman et al. 2000) and Kenny/DmIKK $\gamma$  (Rutschmann et al. 2000b; Silverman et al. 2000) have been recently described in *Drosophila* as homologues of mammalian IKK $\beta$  and NEMO/IKK $\gamma$ , both essentially involved in IL-1R/Toll receptor family-induced NF- $\kappa$ B activation (Karin and Lin 2002). Surprisingly, both molecules have been found to be dispensable for the Toll immune response but have an essential role in the Imd pathway (Table 1), which mediates proteolytical activation of the Rel/NF- $\kappa$ B protein Relish in response to infection with Gram-negative bacteria. However in mammals, a PMA-responsive alternative IKK complex lacking IKK $\alpha$ , IKK $\beta$  and NEMO/IKK $\gamma$  has been implicated in T-cell activation, demonstrating the existence of at least two independent IKK complexes (Peters et al. 2000). This novel IKK complex contains a yet unidentified kinase phosphorylating serine-32 (S32) and serine-36 (S36) of I- $\kappa$ B, and the LPS-inducible IKK-related kinase IKK $\epsilon$ /IKK $\iota$  phosphorylating only S36 of I- $\kappa$ B (Shimada et al. 1999; Peters et al. 2000). There is an additional IKK $\epsilon$ /IKK $\iota$ -like kinase designated as NAK/TBK1/T2 K in mammals that selectively phosphorylates S36 of I- $\kappa$ B and seems to have a role in activation of the "classical" IKK $\alpha$ /IKK $\beta$ /NEMO complex (Pomerantz and Baltimore 1999; Bonnard et al. 2000; Tojima et al. 2000). The *Drosophila* DNA database revealed in addition to DmIKK $\beta$  only one IKK-like kinase, with closest homologies to IKK $\epsilon$ /IKK $\iota$  and TBK/NAK/T2 K. Thus, it is likely that the *Drosophila* homologue of IKK-related kinases may be part of a Cactus kinase complex involved in Toll signaling acting independently from the DmIKK $\beta$ /DmIKK $\gamma$  complex.

## 2.2

### The Imd Pathway in Innate Immunity of *Drosophila*

The *Drosophila* Imd pathway, which is activated in response to Gram-positive bacteria, leads to the activation of Relish, a member of the Rel/NF- $\kappa$ B family (Fig. 4A). In addition to its amino-terminal Rel-homology domain, Relish has six carboxy-terminal ankyrin repeats holding the molecule inactive in the cytoplasm of non-induced cells (Dushay et al. 1996; see Appendix 1). Thus, the inhibitory carboxy-terminal domain of Relish fulfills a similar function as Cactus in the Toll pathway. Activation of Relish relies on the inducible endo-proteolytical cleavage of the molecule, releasing the amino-terminal RHD from the inhibitory ankyrin repeats, thus allowing the translocation of the former into the nucleus (Silverman et al. 2000; Stoven et al. 2000). In mammals, the p52 NF- $\kappa$ B subunit is released in a strikingly similar signal-induced manner



**Fig. 4.** Model of signal-induced processing of Rel/NF- $\kappa$ B precursor proteins A *Drosophila* and B mammalian

from its p100 precursor (Fig. 4B). The link in the Imd pathway between Gram-negative bacteria in the extracellular space and the intracellular signaling pathway leading to Relish processing is based on the peptidoglycan recognition protein PGRP-LC (Gottar et al. 2002; Ramet et al. 2002). PGRP-SA, which triggers the Toll pathway upon infection with Gram-positive bacteria, and PGRP-LC belong to a large family of phylogenetically conserved proteins involved in the recognition of peptidoglycans (Werner et al. 2000; Liu et al. 2001). In contrast to PGRP-SA which is a soluble protein, PGRP-LC is a type II transmembrane protein with three peptidoglycan-binding domains in its extracellular part and a large intracellular domain lacking homology to proteins of known function (Gottar et al. 2002; Ramet et al. 2002). The name-giving Imd protein acts downstream of PGRP-LC and has recently been identified as a cytosolic protein with a carboxy-terminal death domain (Appendix 3) showing significant homology to the death domains of the mammalian adaptor proteins TRADD, FADD and RIP, the latter also exerting serine/threonine kinase activity (Georgel et al. 2001). These mammalian adaptor proteins link death domain-containing cell surface receptors to intracellular signaling cascades and lead to apoptosis-induction and/or NF- $\kappa$ B activation. It is therefore tempting to speculate that Imd also acts as a receptor-proximal adaptor coupling PGRP-LC to Relish activation. Noteworthy, both molecules, Imd and its closest mammalian homologue RIP, have been implicated in activation of Rel/NF- $\kappa$ B members, but also in the induction of cell death (Bodmer et al. 2000; Georgel et al. 2001). The next molecular event in Imd-mediated activation of Relish is activation of the MAP3K DTak1 (Vidal et al. 2001). DTak1 then in turn stimulates the *Drosophila* IKK complex, which phosphorylates Relish and thereby induces its endoproteolytic activation (Rutschmann et al. 2000b; Silverman et al. 2000). In a similar way, the mammalian MAP3 K NF- $\kappa$ B-inducing kinase (NIK) stimulates the IKK complex, which in turn activates the NF- $\kappa$ B2 precursor p100 (Senftleben et al. 2001; Xiao et al. 2001a,b) (Fig. 4B). However, with respect to the proteolytic activities involved in the processing of p100 and Relish, there is a striking difference: p100 is processed via an ubiquitination-dependent process by the proteasome (Heusch et al. 1999) whereas Relish is cleaved by Dredd (Leulier et al. 2000), a caspase otherwise involved in apoptosis induction (Chen et al. 1998). How the IKK complex-activating triple kinases DTak1 and NIK are linked to receptors triggering processing of Relish and p100 is still unclear. Again one possibility is that adaptor proteins of the TRAF family are involved. Indeed, in mammals, both NIK and Tak1 can form a complex with TRAF proteins (Song et al. 1997; Ninomiya-Tsuji et al. 1999). Moreover, the Imd-related mammalian adaptor proteins TRADD and RIP interact with TRAF proteins and members of the TNF receptor family, thereby linking these receptors in a TRAF-dependent manner to the activation of the NF- $\kappa$ B pathway (Wajant and Scheurich 2001). However, an involvement of TRAF proteins in receptor-induced processing of Relish or p100 has not been shown yet and future studies must reveal whether such a connection indeed exists.

### 3

#### **Toll and TRAF-Related Signaling in *Caenorhabditis elegans***

Although putative members of the Toll/IL-1R, Pelle/IRAK, Cactus/I- $\kappa$ B and TRAF protein family have been identified in *C. elegans* (Wajant et al. 1998; Rich et al. 2000; Pujol et al. 2001), other components involved in Toll-induced NF- $\kappa$ B signaling, like the Rel/NF- $\kappa$ B proteins itself or components of the IKK complex, appear to be absent in this species (Pujol et al. 2001). It is noteworthy that none of the aforementioned homologues has been implicated in *C. elegans* resistance against microbial challenge (Pujol et al. 2001). However, a null allele of *C. elegans* Toll (CeToll) has a developmental phenotype whereas putative null alleles of homologues of Pelle/IRAK, Cactus/I- $\kappa$ B and TRAF exert no obvious phenotypes (Pujol et al. 2001). Thus, the molecular basis of developmental signaling by CeToll and *Drosophila* Toll are clearly distinct. In agreement with this difference, an allele of CeToll with a defect in the conserved TIR domain, which in vertebrates and arthropods is involved in coupling Toll receptors to the NF- $\kappa$ B pathway, has only a minor developmental defect (Pujol et al. 2001). Remarkably, worms with the TIR domain-impaired Toll allele have lost their capability to distinguish between different bacterial species and are unable to avoid certain pathogenic bacteria. Again, the *C. elegans* homologues of Toll/IL-1R, Pelle/IRAK, Cactus/I- $\kappa$ B and TRAF are apparently not involved in this process. It is tempting to speculate that CeToll, like some other members of this receptor family, is involved in recognition of specific bacterial patterns. An obvious question is whether *C. elegans* has secondary lost parts of a formerly existing Rel/NF- $\kappa$ B pathway or whether this type of pathway has never evolved in the worm. The existence of proteins related to Toll/IL-1R, Pelle/IRAK, Cactus/I- $\kappa$ B and TRAF argues not necessarily for the presence of a Rel/NF- $\kappa$ B-related pathway in *C. elegans*, as all these proteins have also been implicated in the JNK pathway, which is functional in *C. elegans*. However, I- $\kappa$ B proteins seem to act exclusively as regulators of the NF- $\kappa$ B pathway making the presence of a putative homologue of I- $\kappa$ B in *C. elegans* (Pujol et al. 2001), a very puzzling fact. Future work concerning the functional role of the putative *C. elegans* I- $\kappa$ B homologue will help us to resolve this issue.

### 4

#### **Mammalian TRAF4 and DTRAF1 Are Highly Conserved and Involved in JNK Activation**

Mammalian TRAF4 and its *Drosophila* homologue DTRAF1 are phylogenetically highly conserved, but also comparably distinct from other members of the TRAF family (Wajant et al. 2001). The sequence identity between both proteins is 45%, whereas the closest TRAF4-related human TRAF protein shares only 26% sequence identity (Wajant et al. 2001). Moreover, the expression



pattern of TRAF4 and DTRAF1 are very similar: both molecules are detectable throughout embryogenesis and are predominantly found in undifferentiated cells, in particular neuronal precursors or epithelial progenitor cells (Krajewska et al. 1998; Masson et al. 1998; Preiss et al. 2001). Thus, the high degree of sequence similarity between DTRAF1 and TRAF4 is also reflected by a partially conserved expression pattern, suggesting that these two TRAF proteins fulfill an ancient conserved function in the differentiation of vertebrate and invertebrate cells. The recent analyses of TRAF4-deficient mice revealed a neural tube closure defect as well as malformation of rib, sternum, the spinal column and the upper respiratory tract. The latter is responsible for the increase in pulmonary inflammation found in these mice (Regnier et al. 2002; Shiels et al. 2000). Although DTRAF1 can interact with Pelle, an involvement in *Drosophila* Toll signaling was not confirmed by microinjection experiments in *Drosophila* eggs (Zapata et al. 2000). Similarly, there is no evidence for a major role of TRAF4 in mammalian NF- $\kappa$ B signaling. However, like other TRAF proteins, DTRAF1, as well as mammalian TRAF4, have been implicated in the activation of the JNK pathway. So, DTRAF1 has been identified as a binding partner of Misshapen, a MAP4K involved in the activation of the JNK pathway in flies (Liu et al. 1999). Moreover, TRAF4 interacts with p47phox, a NAD(P)H oxidase adapter subunit, and both synergistically induce oxidant production and JNK activation (Xu et al. 2002). Remarkably, impairment of the JNK pathway in *Drosophila* prevents dorsal closure (Sluss et al. 1996), a phenotype that is closely related with the neural tube closure defect described in TRAF4-deficient mice (Regnier et al. 2002). Despite the high degree of sequence similarity and domain architecture, there is one striking difference between TRAF4 and its *Drosophila* counterpart: in contrast to TRAF4, DTRAF1 has no amino-terminal RING domain (Liu et al. 1999; Preiss et al. 2001). Although the RING domain of TRAF6/DTRAF2 and TRAF2 have been implicated in the binding of effector proteins (Baud et al. 1999; Devin et al. 2001) and in the E3 ligase activity of these proteins (Shen et al. 2001; Brown et al. 2002), the relevance of this difference for TRAF4/DTRAF1-related functions has not been addressed yet.

**Acknowledgements.** This work was supported by Deutsche Forschungsgemeinschaft Grants Wa 1025/3-1 and Sonderforschungsbereich 495 project A5 and Deutsche Krebshilfe Grant 10-1751-Wa 3.

## Appendix 1

### Rel/NF- $\kappa$ B and I- $\kappa$ B Proteins

The nuclear factor kappa B (NF- $\kappa$ B)/Rel protein family comprises a group of phylogenetically conserved inducible transcription factors that act as homo- and/or heterodimers. Activation of Rel/NF- $\kappa$ B proteins can be induced by a huge variety of inducers, including cytokines, e.g. TNF and IL-1, bacterial prod-

ucts, UV radiation and reactive oxygen species. In non-stimulated cells, the Rel/NF- $\kappa$ B proteins are sequestered in the cytoplasm by interaction with proteins of the inhibitor of  $\kappa$ B (I- $\kappa$ B) family. The NF- $\kappa$ B/Rel proteins are characterized by the conserved Rel homology domain (RHD) which mediates dimerization, DNA binding, nuclear localization and interaction with their inhibitory counterparts of the I- $\kappa$ B family. In mammals, five members of the NF- $\kappa$ B/Rel family have been described: RelA/p65, RelB, and c-Rel as well as NF- $\kappa$ B1/p50, which is released from its precursor p105 by a constitutively active proteolytic process, and NF- $\kappa$ B2/p52, which is released from its precursor p100 by induced proteolysis. In *Drosophila*, three Rel/NF- $\kappa$ B are known: Dorsal, Dif and Relish, the latter undergoes inducible proteolysis like NF- $\kappa$ B2. The I- $\kappa$ B proteins are defined by presence of six to seven copies of conserved helical protein domains, the so-called ankyrin repeats. In mammals, the I- $\kappa$ B family is represented by I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$ , I- $\kappa$ B $\gamma$ , I- $\kappa$ B $\epsilon$ , Bcl-3 and the aforementioned precursors of NF- $\kappa$ B1 and NF- $\kappa$ B2, p105 and p100. In *Drosophila*, there are two I- $\kappa$ B proteins, Cactus, and the precursor of Relish which carries six ankyrin repeats in its carboxy-terminal part (Dushay et al. 1996). The key event in activation of Rel/NF- $\kappa$ B transcription factors is the release of these proteins from their inhibitory I- $\kappa$ B counterparts. This release is based on the induced proteolytic degradation of I- $\kappa$ B proteins. In the case of the Rel/NF- $\kappa$ B precursors p100, p105 and Relish, proteolytic action is restricted to the carboxy-terminal ankyrin repeat-containing domain and therefore leads to the release of the active amino-terminal RHD. Proteolytic degradation of I- $\kappa$ B proteins solely consisting of ankyrin repeats as well as the proteolytic processing of RHD – ankyrin repeat chimeric precursors are triggered by phosphorylation of the I- $\kappa$ B proteins by a multi-protein complex designated as I- $\kappa$ B kinase (IKK) complex. The prototypic IKK complex in mammals consists of two related kinases, IKK1 and IKK2, phosphorylating I- $\kappa$ B proteins, a regulatory subunit called NEMO/IKK $\gamma$  and Hsp90 and cdc37 (Chen et al. 2002). There is considerable evidence suggesting the existence of several distinct IKK complexes. This is for example obvious from the phenotype of IKK2-deficient mice, which are still able to form a cytokine-responsive IKK complex, but lack an IKK complex with the ability to promote phosphorylation-dependent processing of the NF- $\kappa$ B2 precursor p100. Moreover, a PMA-inducible I- $\kappa$ B kinase complex has been biochemically defined which lacks IKK1, IKK2 and NEMO, but contains a novel IKK1/2-related kinase, IKK $\epsilon$  (Peters et al. 2000). As homologues of IKK2, NEMO/IKK $\gamma$  and IKK $\epsilon$  have also been described in *Drosophila*, distinct IKK complexes may also exist in this organism.

For more extended reviews concerning Rel/NF- $\kappa$ B and I- $\kappa$ B proteins, see Silverman and Maniatis (2001); Ghosh and Karin (2002); Karin and Lin (2002).

## Appendix 2

### MAP Kinase Cascades

Mitogen-activated protein kinase (MAPK) cascades are phylogenetically conserved signaling modules composed of three sequentially acting kinases: a MAPK, which is activated through threonine and tyrosine phosphorylation by a dual specificity MAPK kinase (MAPKK, MEK, MKK) which in turn becomes activated by a serine/threonine MAPKK kinase (MAPKKK, MEKK). MAPK cascades can be triggered by a huge variety of signals including growth factors, cytokines and physical cues like osmolarity. Consequently, they are critically involved in an extensive number of cellular processes. Three related groups of MAPKs are found from flies to mammals, defining functionally distinct MAPK modules: the extracellular signal-regulated kinases pathway, the c-Jun N-terminal kinases (JNK) pathway and the p38 kinases pathway. While the MAPKs are activated specifically by only one or two MAPKKs, these intermediate kinases are promiscuously activated by the various known MAPKKK. In the activation of the MAPKKK, a plethora of molecules can be involved including GTP-binding proteins, members of the germinal center kinase family of kinases and TRAF adaptor proteins.

For more extended reviews concerning MAP kinase cascades, see Davis (2000); Chang and Karin (2001); Hazzalin and Mahadevan (2002); Shaulian and Karin (2002).

## Appendix 3

### The Death Domain

The death domain (DD) has been originally identified by deletion mutagenesis of the apoptosis-inducing receptor Fas as that part of the molecule which is necessary to transduce the apoptotic signal generated on this receptor. Subsequently, this domain has also been found in other death-inducing receptors, in a variety of cytosolic adaptor proteins and also some kinases. It is currently clear that death domains act as protein-protein interaction modules by homophilic DD-DD association. X-ray structure analyses of several death domains revealed that this domain consists of six antiparallel amphipathic  $\alpha$ -helices arranged in a conserved overall fold. Structural and sequence analyses revealed that the death effector domain (DED) and the caspase-recruitment domain (CARD), two other conserved protein-protein interaction domains implicated in apoptotic pathways, also adopt a fold similar to that of death domains. Remarkably, DDs, DEDs and CARDs interact specifically with members of their own subfamily. Although all three domains are originally identified in homophilic protein-protein interactions involved in apoptosis signaling, they can also be part of non-apoptotic signaling pathways. Recently, a fourth domain, the Pyrin domain, has been identified by bioinformatics, which might also adopt a death domain-related fold.

For more extended reviews concerning the role and function of death domain-containing proteins, see Fesik (2000); Martinon et al. (2001).

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# The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology

## Review

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### Introduction

Three decades ago, lymphotoxin (LT) and tumor necrosis factor (TNF) were identified as products of lymphocytes and macrophages that caused the lysis of certain types of cells, especially tumor cells (Granger et al., 1969; Carswell et al., 1975). When the cDNAs encoding LT $\alpha$  and TNF were cloned (Gray et al., 1984; Pennica et al., 1984), they were similar to one another and, eventually, it became clear that they were members of a gene superfamily. Not surprisingly, the receptors for these proteins also constitute a TNF receptor (TNFR)-related gene superfamily. Large-scale sequencing of “expressed sequence tags” (ESTs) identified many related proteins, collectively referred to here as TNF- and TNFR-related superfamily proteins (TNF/TNFR SFPs; reviewed in Smith et al., 1994; Ashkenazi and Dixit, 1998; Wallach et al., 1999; Idriss and Naismith, 2000; <http://www.gene.ucl.ac.uk/users/hester/tnfinfo.html>). The familiar as well as standardized names of these proteins are listed in Table 1, together with their gene locations, phenotypes caused by mutations in these genes, and identified functions.

The discovery that cachectin, a protein known to cause fever and wasting, was identical to TNF provided an early illustration of the importance of members of this family in human disease (Beutler and Cerami, 1986). Though systemic toxicity dashed early hopes of using LT $\alpha$  and TNF as anti-tumor agents, the discovery of new TNF/TNFR SFPs unveiled new lines of investigation into host defense, inflammation, apoptosis, autoimmunity, and organogenesis. The potent biological effects of TNF/TNFR SFPs participate in human diseases and may be harnessed to ameliorate certain illnesses (Siegel et al., 2000). Pharmaceuticals to inhibit TNF have been developed which control previously recalcitrant inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (Maini and Taylor, 2000; Papadakis and Targan, 2000). Indeed, for reasons we outline below, TNF and other TNF/TNFR SFPs are now being targeted for therapies against widespread human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer.

The receptors and ligands in this superfamily have unique structural attributes that couple them directly to signaling pathways for cell proliferation, survival, and differentiation. Thus, they have assumed prominent roles in the generation of tissues and transient microenvironments. Most TNF/TNFR SFPs are expressed in the immune system, where their rapid and potent signaling capabilities are crucial in coordinating the proliferation and protective functions of pathogen-reactive cells. Here, we review the organization of the TNF/TNFR SF and how these proteins have been adapted for processes as seemingly disparate as host defense and organogenesis. In interpreting this large and highly active area of research, we have focused on common themes that unite the actions of these genes in different tissues. We also discuss the evolutionary success of this superfamily—success that we infer from its expansion across the mammalian genome and from its many indispensable roles in mammalian biology.

### Structure/Function Relationships of TNFRs

The normal functions of TNF/TNFR SFPs, as well as certain diseases involving them, depend on the obligatory 3-fold symmetry that defines the essential signaling stoichiometry and structure (Figure 1). The ligands are type 2 proteins that can have both membrane-embedded “pro” as well as cleaved, soluble “mature” forms (for review, see Idriss and Naismith, 2000). Both forms are active as self-assembling noncovalent trimers, whose individual chains fold as compact “jellyroll”  $\beta$  sandwiches and interact at hydrophobic interfaces (Fesik, 2000) (Figure 1A). The 25%–30% amino acid similarity between TNF-like ligands is largely confined to internal aromatic residues responsible for trimer assembly. The external surfaces of ligand trimers show little sequence similarity, which accounts for receptor selectivity (Figure 2). The ligand shape is that of an inverted bell that is embraced on three sides at the base by elongated receptor chains forming a 3:3 symmetric complex (Figures 1A–1C). Certain ligands and receptors in the TNF/TNFR SFP can bind more than one partner with specific high affinity ( $K_d = 10^{-9}$ – $10^{-10}$  M), thereby enhancing regulatory flexibility and complexity (Idriss and Naismith, 2000). After ligand binding, the receptor cytoplasmic tails form a 3:3 internal complex with signaling proteins such as TRAF2 or FADD (McWhirter et al., 1999) (Figure 1F). Hence, ligand binding and signal complex formation involve stoichiometrically defined protein complexes with 3-fold symmetry. How evolution settled on tri-fold symmetry is unclear. Trimers require more contacts than dimers and may cause an exponential increase in avidity. Trimers could also be necessary to project elongated receptor chains upright on the cell surface. Though few other things in nature occur in threes, trimers provide a unity of design and function for these receptor/ligand superfamilies.

TNFR-like receptors are type 1 transmembrane proteins that adopt elongated structures by a scaffold of disulfide bridges (Figures 1D and 1E). The disulfide

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Table 1. Members of the TNF/TNFR Superfamily

	Standardized	Other Names	Accession	Human Chromosome	Mouse Chromosome	Phenotypes associated with mutations	Additional functional observations
<b>Receptor</b>							
NGFR	TNFRSF16	p75	M14764	17q21-q22	11, 55.6 cM	Defective sensory neuron innervation; impaired heat sensitivity	
Troy	TNFRSF19	Taj	AF167555	13q12.11-12.3	14		Expressed in hair follicles and epithelium; the mouse gene is located near the waved coat locus.
EDAR			AF130988	2q11-q13	10, 29.0 cM	Hypohydrotic ectodermal dysplasia — abnormal tooth, hair and sweat gland formation	
XEDAR		EDA-A2R		AF298812	X		Likely role in skin, hair and tooth formation
CD40	TNFRSF5	p50, Bp50	X60592	20q12-q13.2	2, 97.0 cM	Defective Ig class switching and GC formation causing immunodeficiency	
DcR3	TNFRSF6B		AF104419	20q13			Secreted decoy receptor for FasL with possible role in tumor evasion
FAS	TNFRSF6	CD95, APO-1, APT1	M67454	10q24.1	19, 23.0 cM	Impaired activation-induced T cell death; lymphoproliferation; autoimmunity (ALPS)	
OX40	TNFRSF4	CD134, ACT35, TXGP1L	X75962	1p36	4, 79.4 cM	Defective T cell responses	
AITR	TNFRSF18	GITR	AF125304	1p36.3	4		Glucocorticoid-induced; inhibits T cell receptor-dependent apoptosis
CD30	TNFRSF8	Ki-1, D1S168E	M83554	1p36	4, 75.5 cM		Marker of Reed-Sternberg cells in Hodgkin's disease
HveA	TNFRSF14	HVEM, ATAR, TR2, LIGHTR	U70321	1p36.3-p36.2			Probable role in T cell proliferation and receptor for herpes simplex virus
4-1BB	TNFRSF9	CD137, ILA	L12964	1p36	4, 75.5 cM		Probable role in T cell responses
TNFR2	TNFRSF1B	CD120b, p75, TNFR, TNFR80, TNF-R-II	M32315	1p36.3-p36.2	4, 75.5 cM	Increased sensitivity to bacterial pathogens; decreased sensitivity to LPS; reduced antigen-induced T cell apoptosis	
DR3	TNFRSF12	TRAMP, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3	U72763	1p36.2			A linked, partially duplicated copy of the gene encodes a potential decoy receptor
CD27	TNFRSF7	Tp55, S152	M63928	12p13	6, 60.35 cM	Defective T cell responses	
TNFR1	TNFRSF1A	CD120a p55-R, TNFR, TNFR60 TNF-R-I	M75866	12p13.2	6, 60.55 cM	Impaired clearance of bacterial pathogens; resistance to LPS; LN present; defective GC formation; defective PP formation	
LTβR	TNFRSF3	TNFR2-RP, TNFCR, TNF-R-III	L04270	12p13	6, 60.4 cM	Absence of LN, PP; defective GC formation	
RANK	TNFRSF11A	TRANCE-R	AF018253	18q22.1		Osteopetrosis; absence of osteoclasts; absence of lymph nodes; PP present; abnormal B cell development	Required for lactating mammary gland development
TACI		CAML Interactor		AF023614	17p11	11	Probable role in B cell responses
BCMA	TNFRSF17	BCM	Z29574	16p13.1			Probable role in B cell responses
DR6		TR7	NM_014452	6p21.1-12.2			

(Continued next page)

Table 1. Continued.

	Standardized	Other Names	Accession	Human Chromosome	Mouse Chromosome	Phenotypes associated with mutations	Additional functional observations
<b>Receptor</b>							
OPG	TNFRSF11B	OCIF, TR1 osteoprotegerin	U94332	8Q24		Osteoporosis; arterial calcification	
DR4	TNFRSF10A	Apo2, TRAILR-1	U90875	8p21			Probable inducer of lymphocyte death and activation
DR5	TNFRSF10B	KILLER, TRICK2A, TRAIL-R2, TRICKB	AF012628	8p22-p21			Probable inducer of lymphocyte death and activation
DcR1	TNFRSF10C	TRAILR3, LIT, TRID	AF012536	8p22-p21			GPI-linked decoy receptor—interferes with TRAIL signaling
DcR2	TNFRSF10D	TRUNDD TRAILR4	AF029761	8p21			Transmembrane decoy receptor—interferes with TRAIL signaling
<b>Ligand</b>							
EDA		EDA1	NM_001399	Xq12-q13.1	X, 37.0 cM	Hypohydrotic ectodermal dysplasia—abnormal tooth, hair and sweat gland formation	
CD40L	TNFSF5	IMD3, HIGM1, TRAP, CD154, gp39	X67878	Xq26	X, 18.0 cM	Defective T cell and IgG responses; hyper IgM syndrome	
FasL	TNFSF6	APT1LG1	U11821	1q23	1, 85.0 cM	Impaired activation-induced T cell death; lymphoproliferation; autoimmunity; ALPS	
OX40L	TNFSF4	gp34 TXGP1	D90224	1q25	1, 84.9 cM	Defective T cell responses	
AITRL	TNFSF18	TL6, hGITRL	AF125303	1q23			Inhibits T cell receptor-dependent apoptosis
CD30L	TNFSF8		L09753	9q33	4, 32.2 cM		Possible role in malignant lymphocyte disorders
VEGI	TNFSF15	TL1	AF039390				Potential vascular endothelial cell growth inhibitor
LIGHT	TNFSF14	LT <sub>α</sub> , HVEM-L	AF036581	19 (probable)	17		
4-1BBL	TNFSF9		U03398	19p13.3	17	Defective T cell responses	
CD27L	TNFSF7	CD70	L08096	19p13	17, 20.0 cM		
LT <sub>α</sub>	TNFSF1	TNFB, LT	X01393	6p21.3	17, 19.06 cM	Absence of LN and PP; disorganized splenic microarchitecture; defective GC formation	
TNF	TNFSF2	tumor necrosis factor; cachectin, TNFA, DIF	X01394	6p21.3	17, 19.06	LN present; defective GC formation; increased susceptibility to microbial pathogens	
LT <sub>β</sub>	TNFSF3	TNFC, p33	L11015	6p21.3	17, 19.061	Absence of peripheral LN and PP; presence of mesenteric and some cervical LN; defective GC formation	
TWEAK	TNFSF12	DR3L APO3L	AF030099	17p13	11?		Potential role in monocyte and NK cell cytotoxicity
APRIL	TNFSF13		NM_003808	17p13.1	11?		Probable role in B cell responses
BLYS	TNFSF13B	BAFF, THANK, TALL1	AF132600	13q32-34			Probable role in B cell responses
RANKL	TNFSF11	TRANCE, OPG, ODF	AF013171	13q14	14, 45.0	Osteopetrosis; absence of osteoclasts; absence of lymph nodes; PP present; normal splenic architecture; abnormal B cell and T cell development	Required for lactating mammary gland development
TRAIL	TNFSF10	Apo-2L TL2	U37518	3q26			

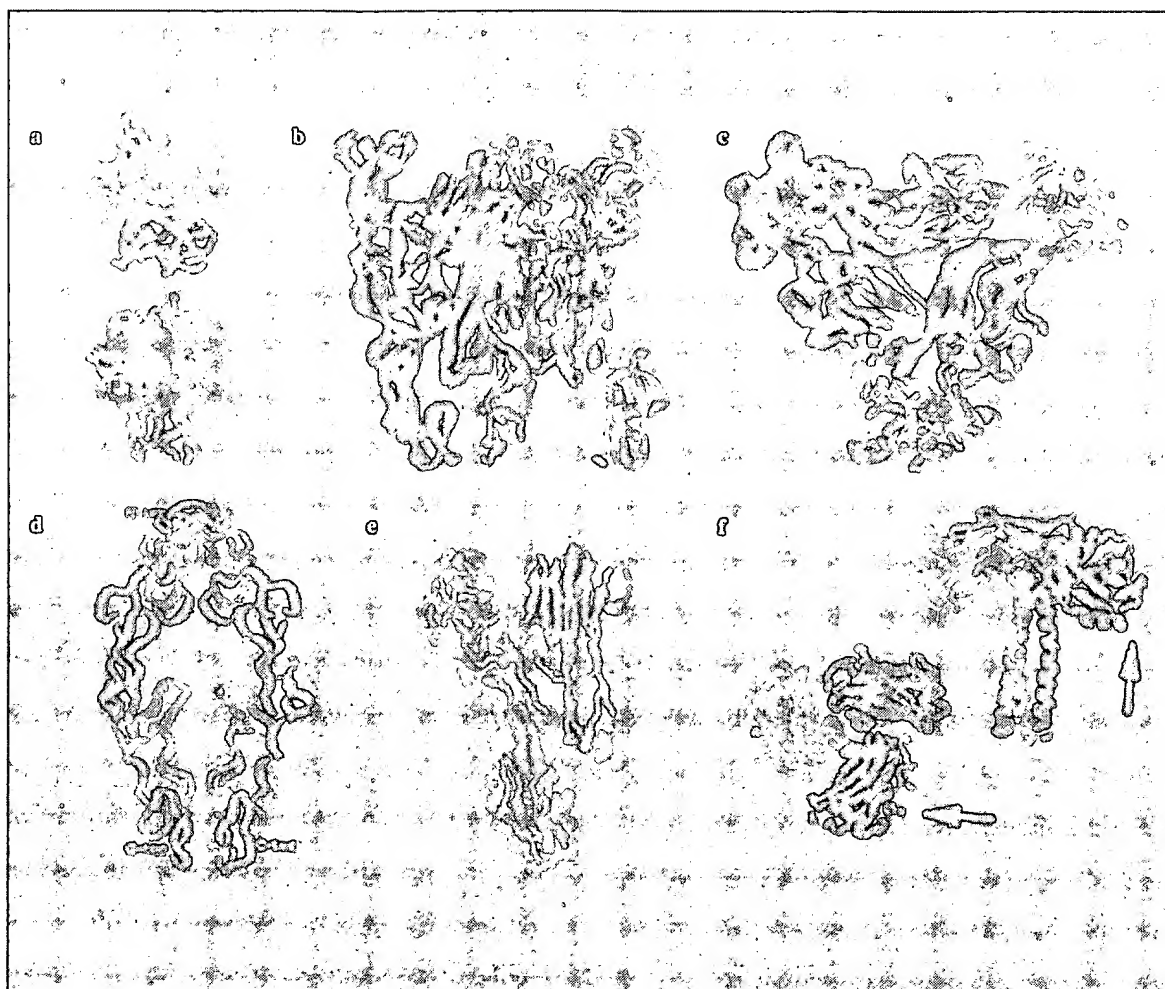


Figure 1. Structure of TNF/TNFR Interaction

- (A) Trimeric structure of TNF. Structure of the TNF trimer shown from the top (upper) and side (lower) views with each monomer differently colored. The  $\beta$ -pleated sheets assume a "jellyroll" orientation in each monomer (adapted from Eck and Sprang, 1989).
- (B) Trimeric symmetry of the structure of the active domain of TNFR1 complexed with LT $\alpha$ . The elongated ladder of disulfide bridges for the receptor chains (light green, blue, and crimson) is highlighted in red. The predominant contacts occur between the trimeric ligand (chains in dark green and brown) and the middle of the ectodomain in the region of CRD2 (Banner et al., 1993).
- (C) LT $\alpha$ /TNFR1 structure from a "top" view looking down at the membrane revealing the tri-fold symmetry in which the receptor chains embrace the ligand at each vertex of a triangle formed by the apposition of ligand monomers.
- (D) Neutral pH structure of the unliganded TNFR1 ectodomain. A parallel dimer with extensive contacts in the PLAD region comprising CRD1 is shown. An anti-parallel dimer, not shown, was also observed (Naismith et al., 1995).
- (E) Structure of the liganded monomeric complex. Contact structure modeled on the interaction between a monomer of LT $\alpha$  and a monomer of TNFR1 showing that the ligand contacts are made primarily in CRD2 but not CRD1 (Banner et al., 1993).
- (F) The trimeric structure of TRAF complexes with CD40 peptides. The three TRAF monomers (light blue, dark blue, and green) extensively contact each other and interact at the tips of the globular N-terminal domains with the cytoplasmic portions of receptor monomers as illustrated by the position of receptor peptides (yellow or gold) indicated by the arrows (McWhirter et al., 1999).

bonds form "cysteine-rich domains" (CRDs) that are the hallmark of the TNFR superfamily (e.g., see alignments of amino-terminal CRDs in Figure 3). These 40 amino acid pseudorepeats are typically defined by 3 intrachain disulfides generated by 6 highly conserved cysteines (Smith et al., 1994). The elongated receptor chains fit in the "grooves" between protomers within the ligand trimer. For Fas and TNFR1, ligand contacts occur mainly in the 2nd and 3rd CRDs (counted from the N terminus). The crystal structure of LT $\alpha$  in complex with the TNFR1 extracellular domain reveals no contact between individual receptor chains (Figures 1C and 1D) (Banner et

al., 1993). From this it was inferred that the ligand recruited or "cross-linked" three receptor monomers into the final 3:3 complex. This view has been recently challenged by findings that several receptors in the TNFR family self-assemble in the absence of ligand and signaling involves rearrangement of the preassembled chains (Chan et al., 2000a; Siegel et al., 2000). The structure of TRAIL complexed with its receptor DR5 reveals a remarkable conservation of the same 3-fold ligand-receptor complex as seen for LT $\alpha$ /TNFR1 despite a minimum of primary sequence similarity (Hymowitz et al., 1999).



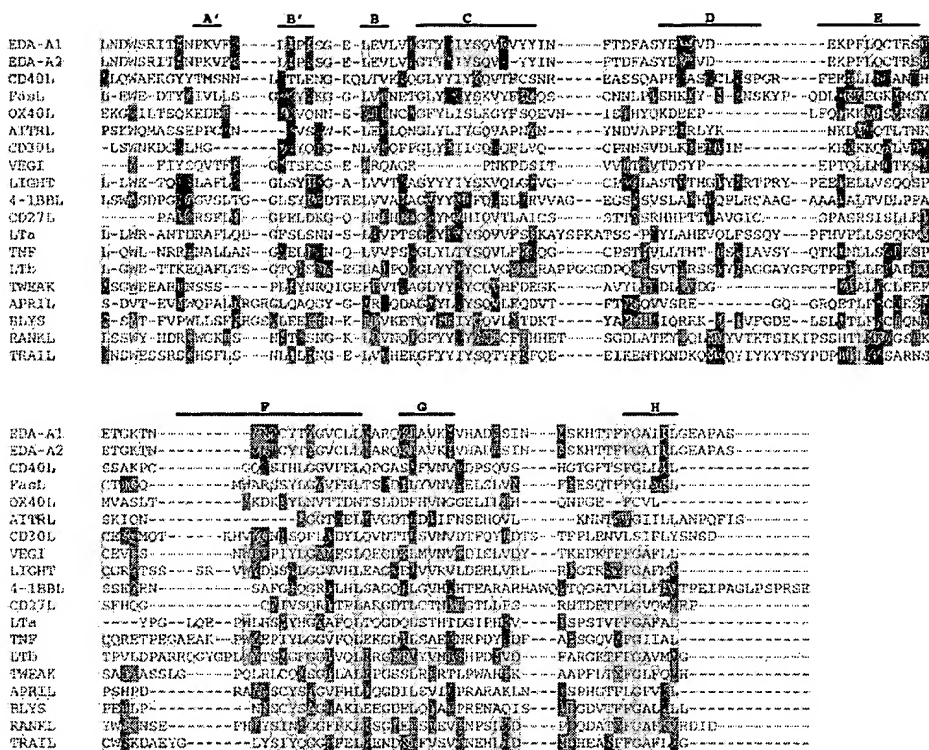


Figure 2. Sequence Alignment of the TNF-Related Proteins

Residues are shaded (yellow for identities, red for conservative substitutions) where they occur in 30% of the grouped sequences. Green shading highlights the corresponding locations of intron excision sites from the mRNAs that encode the proteins. The locations of  $\beta$  strands in the LT $\alpha$  structure are shown at the top of the alignment. Alignments were generated using CLUSTALW.

TNFRs can display noncanonical functions that do not require binding trimer ligands. For example, NGFR binds neurotrophins, ligands with no structural resemblance to TNF, and interacts with the Trk family of tyrosine kinase receptors that are unrelated to TNFRs. Thus, the *NGFR* gene, with a dissimilar distribution of introns, may have been formed early in the evolution of the TNFR SF. HveA, formerly HVEM, another TNFR SFP, is a receptor for Herpesvirus, type 1, and binds the TNF-like ligand, LIGHT (Mauri et al., 1998). Similarly, the avian TNFR-like TVB(S1), TVB(S3)(CAR-1), and TVB(T) molecules are receptors for avian leukemia viruses (Adkins et al., 2000). Whether these functions require preassembly and receptor trimers are subjects of ongoing research.

#### Two Pivotal Modes of Signaling

The cytoplasmic domains of TNFRs are modest in length and function as docking sites for signaling molecules. Signaling occurs through two principal classes of cytoplasmic adaptor proteins: TRAFs (TNF receptor-associated factors) and "death domain" (DD) molecules (reviewed in Fesik, 2000; Inoue et al., 2000). In mammals, at least six TRAF molecules and a number of nonreceptor DD molecules have evolved at locations spread through the genome (Wajant et al., 1999). The signaling adaptor is selected by whether the cytoplasmic domain of the receptor harbors either a DD or a TRAF binding motif. The DD is a roughly 60 amino acid globular bundle of 6 conserved  $\alpha$  helices found in the receptor tail and the

adaptor that promotes homotypic association (Figure 4). By contrast, the TRAF binding motif is a stretch of amino acids (less than a dozen contact residues) in the receptor tail that is clutched by a pocket in the globular head group of the adaptor through charged residues (Figure 1F). Signaling is extremely rapid and highly specific. For the subset of receptors that have DDs, often called "death receptors" (DR), ligand engagement typically causes the association of adaptors such as Fas-associated DD protein (FADD) and TNFR-associated DD protein (TRADD) that ultimately cause caspase activation and cell death. For Fas, the homotypic association of FADD with the Fas DD leads to the recruitment of caspase-8 or -10 by homotypic interactions between "death effector domains" (DEDs) contained in FADD and the prodomain of these two caspases (Scaffidi et al., 1999).

Why adaptors? An obvious answer is that modularity allows regulatory flexibility. The DD, DED, and "caspase-recruitment domain" (CARD), despite only 10%–20% sequence identity, all share the same overall 6  $\alpha$ -helical structure (Fesik, 2000). This suggests a common origin from a prototype molecule that became specialized for roles at different points in the signaling pathway. For example, whereas DD and DED play essential associative roles in death receptor pathways, the CARD has been diversified for mitochondrial death pathways and inflammatory responses (Fesik, 2000; Humke et al., 2000). The DD also resembles the "ankyrin" repeat, an oligomerization domain common to other signaling sys-

## CRD1a

```

CD40  CS--EAY--LINSQCSL---CQPGQKLVSDTE-FETEC
Hv9a  CS--EEDY--PVSECCPK---CSGGRVKEAAGE-LSQTC
Cx40  CV--GDTY--PSNDRCCHE---CRGNGVSRCSR-SQHTC
LTER  CDQDEKVEYE-PQHRICCSH---CPGTYVSAKCSR-IRDTY
RANK  QTS-EPHY---EHLGRCCNK---CEPKYVSSKCTT-TSDYC
TNFR1  CS--LEFYD-CTQKCCSK---CSPQGHAKVCTK-TSDYC
NGFR  CP--TGLY---TSGGCCRA---CNLGECAQFCG--ANQTC
CD27  CP--EPHY-W-AQSKICCOM---CEPGLLVKDCQHEKAAC
CD30  CHGNPSHYD-KAVRCCYR---CPMGLPTQCCPQ--RPTDC
DR3    CP--CAGDHK-KTILFCRG---CPAGHKUKAPCEPCGHTC
TNFR2  CP--SGKYIHPQNSICCTK---CHKETLYLND-CPGPGQDTC
A1TR  CGP-GRLLGTGTDAKCRVHTTRCRDYPGEECCS---ENDC

```

## CRD1b

```

DcR1  VPQQTVAPOQRHSFKGEECPAGSHRSEITGAC
DcR2  VPQQTVAPOQRHSFKGEECPAGSHRSEITGAC
DR4    HDQSEGTQWEHSLPLGELCPPGSHRSEIPGAC
DR5    APQQRABDQKSSPSEGLCPPGHHSIEDGRDC

```

## CRD1c

```

OEG  PPKYLHYDEETSHQLD-DKCPGQTMKQSCAKW-KTIV
DR6  IGTVRYHVDRAFGQVLT-DKCPAGTYVSEHCNTS-LRV
DcR3  ---YPRDDETGERLTC-AQCPGTYVQRPERRDS-PTTC
4-1bb  ---FETRSIQDPC-SNCPAGTE---CDNRR-NQCC
Fas  ---NLGLHHCQFCCHKCPGGERKARDCTVNGDEPDC

```

## CRD1d

```

Troy  CRQDEERDRS-GNCPVPCNO-CGPGMELCECCGEGY-GEDACC
XEDAR  CGENEYWDOW-GRVTCQR-CGPGQELSKDCGYGE-GGDAYC
EDAR  CGENEYWNITGLCOEPP-CGPGCEPYLCCGYGKDEDDYC
BOA  CGENEYHDSLHACPPQLRCS-NTPLTLC---CRYC
TACI  CPEEYWDPELGTCSCKTICNH-QS-QRTS-----AACG

```

Figure 3. Sequence Alignments of the Amino-Terminal Cysteine-Rich Domains of TNFR-Related Proteins

Residues are shaded (yellow for identities, red for conservative substitutions) where they occur in 30% of the grouped sequences. Sequences are assigned to 4 groups according to the organization of the cysteine residues they contain. Alignments were generated using CLUSTALW.

tems (Feinstein et al., 1995). Typically, these domains are encoded by a single exon, a property that has been conserved from *Drosophila* to mammals, suggesting a genetic unit specialized in evolution. These domains self-associate in a manner which can be blocked by selective binding molecules (Humke et al., 2000; Siegel et al., 2000). How the receptor complex activates downstream signaling pathways is not completely clear. The

precise pathways for activation of caspases, NF- $\kappa$ B, and other cellular responses involve a variety of kinases such as p38 and JNK, sphingomyelinase,  $Ca^{2+}$ , and other specialized signaling proteins (Wajant et al., 1999; Idriss and Naismith, 2000). Novel functions continue to emerge, such as the recent finding that TRAF6 can function as a nonproteolytic E3-like ubiquitin ligase implicated in NF- $\kappa$ B activation (Deng et al., 2000). Intense investigation is ongoing to clarify these pathways.

Recent evidence has shown that TNFR chains preassemble into complexes on the cell surface prior to ligand binding (Chan et al., 2000a; Siegel et al., 2000). The formation of oligomers, possibly trimers, in the absence of ligand requires the amino terminal end of the receptor including the first CRD of Fas, TNFR1, and TNFR2. This region, termed PLAD for "pre-ligand assembly domain", is necessary and sufficient for the self-assembly. Parallel dimer structures resulting from the crystallization of the unliganded TNFR 1 ectodomain show extensive contacts in the PLAD region (Figure 1D) (Idriss and Naismith, 2000). The PLAD is distinct from the ligand binding domain and the unliganded complex that it promotes is in a "closed" conformation that is distinct from the 3:3 ligand:receptor assembly. The PLAD interactions are highly specific and usually only receptor homotrimers are formed, however, "transplanting" the TNFR1 PLAD onto TNFR2 allows it to enter TNFR1 complexes. Receptor preassembly is essential for ligand binding and signal transmission. The homotypic domain associations that form the essence of receptor function represent key molecular targets for pharmacological modulation either outside the cell (PLAD, ligand-receptor) or inside the cell (DD, TRAF and DED). The ligands can also preassemble into trimers on the cell surface, and several reports suggest that membrane-anchored ligands can send "reverse" signals into the ligand-bearing cells when they contact their receptor, introducing the possibility of two-way signal transmission which warrants further exploration.

#### TNF/TNFR SFPs Are Cellular Organizers in Metazoans

TNF/TNFR SFPs are communicators between cells and can orchestrate permanent multicellular structures such as lymphoid organs, hair follicles, and bone, as well as impermanent, but long-lived, structures such as the lactating mammary gland. Even more evanescent structures, inflammatory foci, are assembled and disassembled

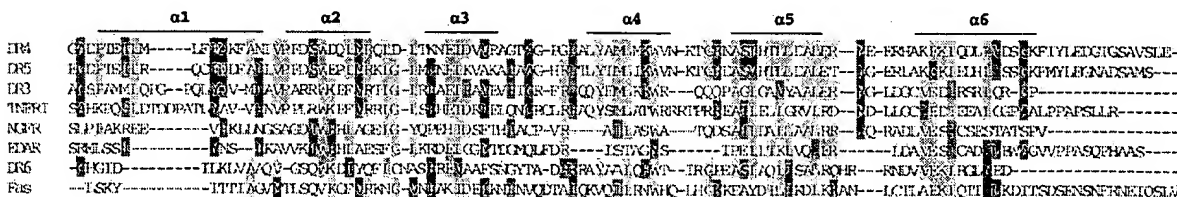


Figure 4. Death Domain-Related Sequences in TNFR SF Proteins

The figure shows an alignment of death domain-related sequences found in the cytoplasmic regions of the indicated TNFR SF proteins. The locations of  $\alpha$  helices in the Fas death domain sequence are indicated at the top; these coincide closely with the location of  $\alpha$  helices in the NGFR structure. Residues are shaded (yellow for identities, red for conservative substitutions) where they are common to half or more of the sequences. Alignments were generated using CLUSTALW.

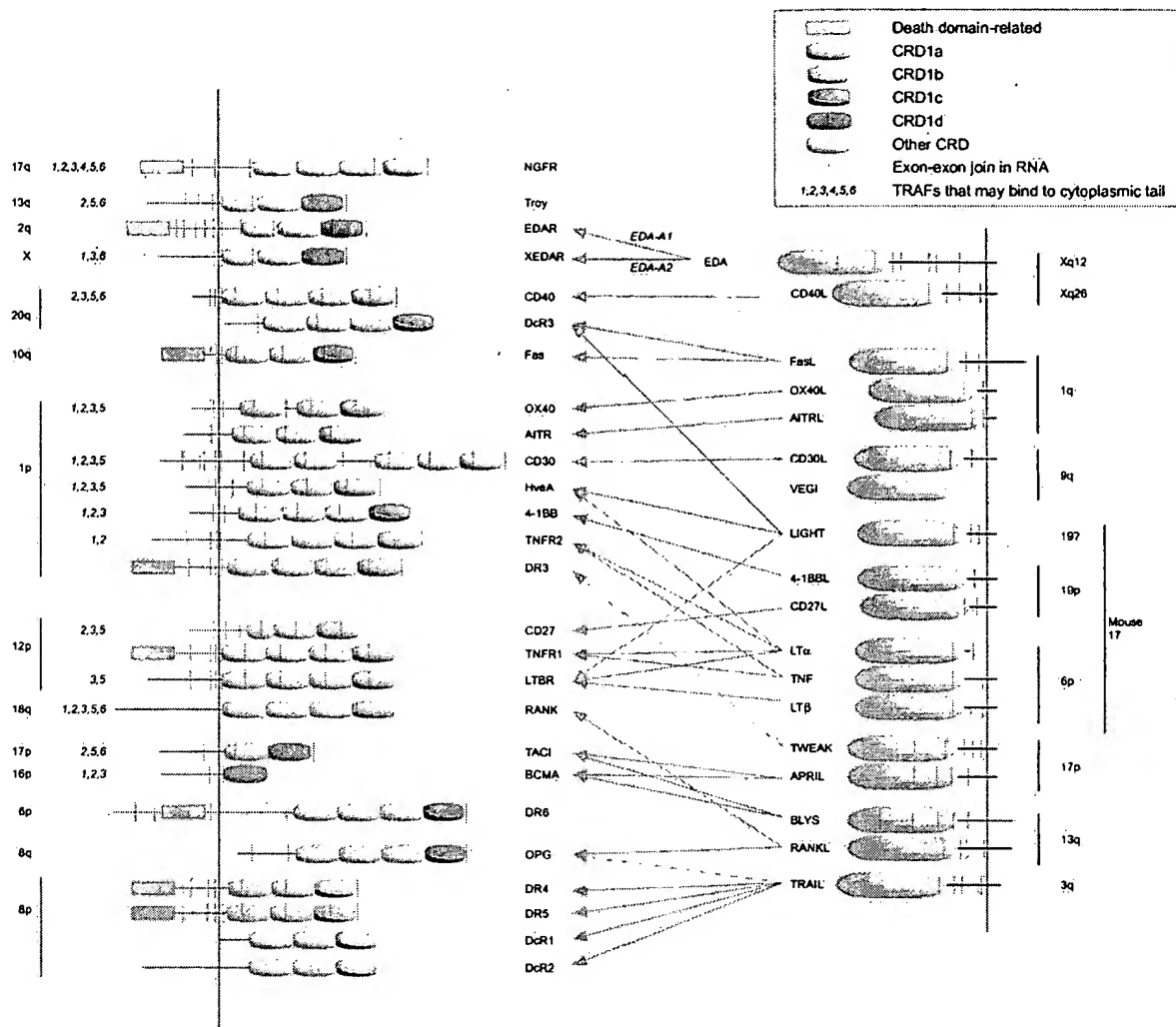


Figure 5. Interacting Proteins of the TNF/TNFR Superfamily

TNFR- and TNF-related proteins are shown at the left and right of the figure, respectively, with arrows connecting ligand-receptor pairs. Cysteine-rich domains (CRDs) are shown as small ovals. The amino-terminal CRDs (CRD1a, 1b, 1c, 1d) are grouped on the basis of sequence similarity (Figure 3) as indicated by the use of colors in the figure. Small vertical lines denote the locations of intron excision sites from the RNAs that encode the proteins (this information was not available for RANK, DcR1, and DcR2). Red boxes mark the locations of death domain-related sequences in the cytoplasmic regions of the TNFR-related proteins. Numbers to the immediate left of the TNFR cytoplasmic regions denote known or inferred interactions with the indicated TRAFs. The locations of the human genes that encode the proteins are provided at the extreme left and right of the figure; the mouse cluster on chromosome 17 is also noted. Potential interactions between TWEAK and DR3, and between TRAIL and OPG, are controversial and are therefore represented by dashed arrows.

bled at limited times and locations in acute responses to infectious stimuli. Broad regulatory patterns involving specific TNF/TNFR SFPs emerge from these examples. The genomic loci and receptor-ligand relationships of the TNF/TNFR SF are depicted in Figure 5.

#### TNF/TNFR SFPs in the Immune System—Coordinating Structure and Response

The major evolutionary advance represented in the vertebrate immune system is a system of "adaptive" or antigen-directed immunity in which TNF/TNFR SFPs play central roles. This involved acquisition of the two recombinase-activating genes, RAG1 and RAG2, ~450 million years ago, as well as adapting perhaps more

ancient somatic mechanisms to mediate recombination and mutation of the combinatorial T cell receptor and B cell receptor genes. Immune receptors thereby diversify during differentiation so that a potentially huge repertoire of B and T lymphocytes (~10<sup>9</sup>), each typically bearing only a single receptor, is generated to ensure responsiveness to a large universe of antigens. Powerful as they are, however, lymphocytes cannot act individually. TNF/TNFR SFPs coordinate the social context of cells that enables lymphocytes to maximally respond to pathogens. Cross-species comparisons suggest that the largest expansion of new members of the TNF/TNFR SF may have occurred during the refinement of the adaptive immune system (see below).

Immune responses occur most effectively within secondary lymphoid organs—aggregates of lymphocytes, antigen-presenting cells, and other immune-responsive cells positioned throughout the body with strategic vascular connections to portals of infectious antigen entry. Remarkably, mice that are genetically deficient for  $LT\beta$  ( $\alpha 1\beta 2$ ) or its receptor  $LT\beta R$  do not develop secondary lymphoid organs, such as lymph nodes or Peyer's patches, and have defective spleen structure and humoral immunity (Fu and Chaplin, 1999). Lymph node anlage, developing at embryonic day (E) 10.5 in the mouse, is formed by invagination of mesenchymal connective tissue constituents expressing  $LT\beta R$ .  $CD4$ -expressing hematopoietic cells seed the anlage and express lymphotoxin ( $LT\alpha 1\beta 2$ ) which stimulates colonization by mature lymphocytes. Deletion of  $RANKL$  or  $RANK$  results in the absence of all peripheral and mesenteric lymph nodes but Peyer's patches remain intact and splenic architecture is unaffected (Dougall et al., 1999; Kong et al., 1999b; Kim et al., 2000a). Requirements for  $RANKL$ / $RANK$  and  $LT\alpha 1\beta 2$ / $LT\beta R$  do not complement one another. Whereas the latter are distributed on both hematopoietic and stromal cells,  $RANKL$  and  $RANK$  are expressed mainly on  $CD4^+$  hematopoietic precursor cells. This suggests that  $CD4^+$  precursors emigrating into the lymph node anlagen use  $RANKL$ / $RANK$  as an autocrine survival/differentiative signal until  $LT\alpha 1\beta 2$  is produced and allows stromal interactions via  $LT\beta R$  to complete node maturation (Kim et al., 2000a). Plasticity in lymphoid organ boundaries is shown by ectopic expression of  $LT\alpha 3$  or  $LT\alpha 1\beta 2$ —or of B cell chemokines induced by  $LT\alpha 3$ / $LT\alpha 1\beta 2$  on B cells—which stimulates vascular adhesion molecules typical of specialized lymph node endothelia and generates ectopic lymphoid tissue (Kratz et al., 1996; Cuff et al., 1999; Luther et al., 2000). Conversely, mice deficient in  $LT\beta R$  or its ligands develop perivascular T cell and B cell infiltrates in multiple tissues (Banks et al., 1995; Alimzhanov et al., 1997). Hence, an ordered, sequential process of  $TNF/TNFR$  SFPs promotes generative interactions between hematopoietic and mesenchymal cells, and establishes spatial constraints essential for lymphoid organ definition (Nishikawa et al., 2000).

#### Participation in Acute Immune Responses

Superimposed on the architecture of lymphoid organs is the coalescence of follicles and germinal center (GC) reactions facilitated by  $TNF/TNFR$  SFPs during active immune responses. These are cellular aggregates comprising mostly B cells but also T cells and follicular dendritic cells (FDCs) (Cyster et al., 2000). FDCs are specialized mesenchymal cells that collect antigens in draining lymph nodes, interact with clonally expanding B cells, and form networks in the follicle under the influence of  $TNF$ ,  $LT\alpha$ ,  $LT\beta$ ,  $TNFR1$ , and  $LT\beta R$  (Fu and Chaplin, 1999). In the spleen, 3% of the total volume of the organ can be occupied by B cell-rich GCs during the peak of an antigen response, yet these GCs will largely disappear by 4 weeks. The residue is a collection of memory lymphocytes that respond more efficiently to previously encountered antigens. In GCs, B cells are stimulated and somatically hypermutate their antigen receptor genes; those with better antigen avidity are selected and can undergo heavy chain class switching to produce differ-

ent antibody subclasses. These processes depend on antigen stimulation followed by engagement of  $CD40$  on the B cells by  $CD40L$  on T cells.  $CD40$  or  $CD40L$  deficiency impairs  $CD4^+$  T cell priming, FDC differentiation, GC formation, and class switching—so IgM-expressing cells cannot undergo isotype conversion to IgG expression, leading to hyper IgM syndrome (see below) (Grewal and Flavell, 1998). Administration of neutralizing anti- $CD40L$  in mature mice causes the abrupt disappearance of existing GCs, revealing the need for tonic  $CD40/CD40L$  interactions. Mice deficient for  $LT\alpha$ ,  $LT\beta$ ,  $TNF$ , and  $TNFR1$  have severe defects in follicle and GC formation (Fu and Chaplin, 1999). Recently, BlyS from activated dendritic cells has been found to interact with the  $TACI$  and  $BCMA$  receptors on B cells and promote their survival (Moore et al., 1999; Laabi and Strasser, 2000) (Table 1). Transgenic mice overexpressing BlyS develop increased B cell numbers and autoimmunity (Mackay et al., 1999). Conversely,  $TACI$  blockers inhibit antibody production and GC formation in normal mice as well as abrogate autoantibody formation and fatal immune complex-mediated nephritis in autoimmune-prone mice (Gross et al., 2000; Laabi and Strasser, 2000; Yan et al., 2000a).

T cell activation is also regulated by  $TNF/TNFR$  SFPs. Except for a partial block in early thymocyte development in the absence of  $RANKL$  (Kong et al., 1999a), effects of  $TNF/TNFR$  SFPs in thymic development,  $CD4/CD8$  lineage commitment, or  $Th1/Th2$  differentiation are minimal. However, the initiation of an immune response by sentinel dendritic cells originating in epithelial barriers stimulating naive T cells in draining lymph nodes involves  $TNF/TNFR$  SFPs. Both  $TNFR$  and  $Fas$  can experimentally costimulate T cell activation, but this effect requires further examination in a physiological setting (Siegel et al., 2000). Additional  $TNF/TNFR$  SFPs, including  $OX40$ ,  $CD27$ , and  $4-1BB$ , regulate the expansion and survival of  $CD4^+$  and  $CD8^+$  T cells responding to dendritic cells that express their respective ligands;  $LIGHT$  may have a similar role (Tamada et al., 2000). Thus far, targeted deletions of these molecules have less drastic consequences on the immune response than does  $CD40/CD40L$  (Table 1). Most  $TNF/TNFR$  SFPs are expressed in activated T cells suggesting that critical immune functions for additional family members await discovery.

#### $TNF/TNFR$ SFPs as Mediators of Cell Death

The capacity to induce cell death is one of the unique properties with great adaptive value that  $TNF/TNFR$  SFPs have evolved. Among the 8 homologous death receptors (DRs) in the  $TNFR$  superfamily (Figure 4), at least 6 can stimulate apoptosis through activation of a family of cysteine proteases called caspases (Raff, 1998; Screaton and Xu, 2000). Other  $TNF/TNFR$  SFPs that lack death domains can potentially modulate the response to DRs or directly influence cell survival. For example,  $TNFR2$  markedly enhances  $TNFR1$ -induced T cell death and  $CD40$  can augment  $Fas$ -induced B cell death (Garone et al., 1995; Chan et al., 2000b). One major function of DR-induced death is cell-mediated cytotoxicity in response to infectious agents.  $Fas$ -mediated cytotoxicity is the major calcium-independent killing mechanism of

CD8<sup>+</sup> cytotoxic T cells (Nagata and Golstein, 1995). Another crucial function of DR-mediated death is immune homeostasis to balance recurrent lymphocyte expansion in response to antigen within the limited space of lymphoid organs. Activated cells must be destroyed, sometimes in enormous numbers. High or repeated antigen stimulation of activated T cells induces these death molecules and causes apoptosis in a fraction of the expanding cell population. This negative feedback mechanism, termed *propriciodal* regulation, prevents the toxic effects of massive lymphocyte expansion (reviewed in Lenardo et al., 1999). Genetic impairment of Fas-induced apoptosis in humans (ALPS) or mice (*lpr/gld*) causes a dramatic loss of lymphocyte homeostasis and autoimmunity (Rieux-Laucat et al., 1995; Lenardo et al., 1999). Self-tolerance mechanisms also involve TNF and dysregulation of TNF has been associated with human systemic lupus erythematosus and other autoimmune conditions (Jacob et al., 1991).

Much is known about how TNF/TNFR SFPs program cell death, but new concepts are still emerging. Cell death has been conceptually divided into two principal forms: apoptosis (programmed cell death) and necrosis (traumatic cell death) (Raff, 1998). There are key morphological differences—apoptosis causes shrinkage, compaction, and breakdown of the cell into easily phagocytosed bits whereas necrosis involves swelling or bursting of the cell with organelle degeneration and loss of plasma membrane integrity. Molecularly, apoptosis involves evolutionarily conserved pathways of caspase activation, but necrosis is less well defined (Raff, 1998). Death receptors within the TNFR SF are among the best-understood inducers of caspase activation and apoptosis. This appeared ironic given the moniker “tumor *necrosis* factor.” However, apoptosis is unable to explain all the death capabilities of these receptors. Death receptor killing in cells without caspase activation and other attributes of apoptosis have now been carefully documented (Kawahara et al., 1998; Vercaamen et al., 1998; Villunger et al., 2000). One important example is vaccinia virus. Although the virus encodes a potent caspase inhibitor, infected cells are nonetheless killed by TNF in a manner that does not resemble apoptosis molecularly or morphologically (Li and Beg, 2000). For lack of a better term, these examples have been called necrosis and may be associated with inflammation. Inflammation is not typically found with apoptosis, but may be extremely important for activating the immune system when TNF-induced necrosis is part of an anti-pathogen response. Conversely, purely apoptotic pathways may be involved in tissue remodeling during development, where inflammation could be detrimental. Experiments dissecting TNFR-induced apoptotic and necrotic pathways may uncover fundamental pathways used by these ligand/receptors in development and host defense.

#### Organizing Reversible Microenvironments in Response to Acute Environmental Perturbations—Acute Inflammation as a Paradigm

The role of TNF/TNFR SFPs in acute inflammation illustrates how they achieve dramatic cellular change and dynamic tissue remodeling. Host defense relies on the

rapid recruitment of many inflammatory cell types to the site of an infection. When the infectious danger has been contained, it is essential that inflammation subsides and normal tissue homeostasis is restored. Rapid response and rapid subsidence are crucial to prevent damage to the host by microbial replication or persistent inflammation, respectively.

Of the many TNF/TNFR SFPs that affect inflammation, TNF has a prominent role. TNF secretion can be induced by conserved structural elements common to microbial pathogens, including cell wall moieties such as peptidoglycan, lipopolysaccharide (LPS), and bacterial DNA CpG motifs, that are bound by Toll-like receptors (TLRs) (Aderem and Ulevitch, 2000). TLRs, conserved in protein sequence from *Drosophila* to humans, decorate epithelial cells, tissue macrophages, and dendritic cells, of which the latter two are the sentinel phagocytic and antigen processing cells of the immune system, respectively. TLRs transcriptionally induce proinflammatory cytokines, including TNF, through the convergence of NF- $\kappa$ B and NF-AT activating pathways, and enhance translational efficiency by a mechanism targeting consensus 3'-untranslated AU-rich elements (ARE) in mRNA (Dumitru et al., 2000). IL-1R, a receptor that shares cytoplasmic homology with TLRs, and integrin interactions with extracellular matrix proteins activate similar pathways, thus amplifying the response (Shornick et al., 1996; de Fougères et al., 2000). The result is a highly complex biologic cascade—involving chemokines, cytokines, and the induction of endothelial adhesins—that recruits and activates granulocytes, monocyte/macrophages, and lymphocytes at the damaged or infected tissue sites. Local injection of TNF recapitulates these events, and TNF- and TNFR-deficient mice show attenuated contact hypersensitivity to irritants and susceptibility to diverse microbial pathogens (Pfeffer et al., 1993; Rothe et al., 1993; Erickson et al., 1994; Pasparakis et al., 1996).

Studies of how TNF mRNA stability is regulated by the presence of an ARE sequence in the 3'-untranslated region may provide insights into the role of TNF/TNFR SFPs and lymphokines in disease. The ARE is common to many cytokine mRNAs and is bound by tristetraprolin (TTP), a zinc finger-containing protein that accelerates the turnover of ARE-containing mRNAs (Carballo et al., 1998). TTP is induced by TNF as a negative feedback loop that limits TNF activity. Mice lacking TTP develop spontaneous, chronic, TNF-induced inflammation, with cachexia, arthritis, and high-titers of anti-DNA antibodies dependent upon TNFR1 (Taylor et al., 1996). Mice containing TNF genes with a deleted ARE sequence are similar, although inflammatory bowel disease also occurs (Kontoyiannis et al., 1999). Mice deficient in TRAF2 (Nguyen et al., 1999) or the TRAF2-interacting zinc finger protein, A20 (Lee et al., 2000), also develop states of TNF excess, suggesting that TRAF2-dependent pathways are involved in negative feedback, perhaps through activation of p38 and JNK (Kontoyiannis et al., 1999).

Spatial and temporal constraints on inflammation by TNFR/TNF SFPs are imposed by feedback inhibition; regulated expression of receptors; soluble processing of membrane-tethered ligands and receptors into soluble forms; and the induction of nonsignaling decoy recep-

tors. Perhaps the most innovative form of regulation for both ligands and receptors of the TNF/TNFR SFP is the proteolytic release of soluble bioactive oligomers from membrane-bound precursors. For TNF, this is accomplished by TACE, a *cis*-acting membrane protease of the metalloprotease/disintegrin/cysteine-rich family, whose regulation is currently obscure (Blobel, 1997). Release of TNF systemically may promote viral infection and cause other pathogenic effects in viral diseases such as AIDS (Fauci et al., 1991). Cleavage from the membrane likely expands the range over which ligands can act, but may also be responsible for more specific effects. For example, stimulation of TNFR2 for certain functions may occur primarily through membrane-bound, but not soluble, TNF (Idriss and Naismith, 2000). Also, lymph node development is predominantly triggered by membrane-bound, but not soluble, LT (Fu and Chaplin, 1999). TACE and/or related proteases cleave TNFRs, thus generating soluble receptors capable of inhibiting TNF. Early lethality in TACE-deficient mice suggests that proteolytic release is a developmentally crucial process that has been subserved by the TNF/TNFR SFPs (Peschon et al., 1998). Some TNFR members, for example, DcR3 and OPG, have evolved a soluble function by loss of a transmembrane domain, whereas others, for example DcR1 and DcR2, can be membrane-tethered but lack a functional death domain. Although regulation through cleavage of TNF/TNFR SFPs remains a plausible but unproven regulatory mechanism, constitutional inflammation and fever in certain cases of TNF receptor-associated periodic syndrome (TRAPS, see below) may be due to defective proteolytic shedding of TNFR1 (Galon et al., 2000). Genetically engineered soluble forms of TNF/TNFR SFPs have been widely used for both research and clinical applications. Typically, fusions with immunoglobulin or leucine zipper oligomerization domains are used to achieve high-level activity of the soluble ligand or receptor (Haak-Frendscho et al., 1994; Walczak et al., 1999). Inflammatory conditions such as arthritis, inflammatory bowel disease, and TRAPS have been successfully treated with soluble TNFR2-Ig fusion protein constructs (Galon et al., 2000).

Left unregulated, TNF can cause chronic inflammation, generalized wasting and, at high levels, septic shock (Idriss and Naismith, 2000); lymphotoxin can induce acute virus-induced shock (Puglielli et al., 1999). Other TNF/TNFR SFPs may be similarly involved in disease pathogenesis. CD40 is induced on vascular endothelial cells in response to TNF (Phipps, 2000). Ligation of endothelial CD40 by CD40L, either expressed on activated monocytes or T cells, or disgorged from platelet granules after activation, produces various inflammatory cytokines, chemokines such as MCP-1, procoagulant activity, adhesion molecules, matrix-degrading metalloproteinases, and inflammatory lipid mediators. Atherosclerotic plaques formed in this milieu are populated by lipid-laden macrophages derived from MCP-1-recruited blood-borne monocytes. Normally, the plaque microenvironment will dissolve upon loss of chronic CD40-CD40L-mediated signals, exemplifying the inherent reversibility of TNF/TNFR SFP-induced neo-tissues. However, TNF or other inflammatory stimuli may promote plaque extension. Interventions that disrupt CD40-

CD40L in experimental models of atherosclerosis both interrupt progression of lesions and change their composition to a more stable, collagen-anchored, character (Lutgens et al., 1999). Patients with unstable angina, a condition associated with risk of plaque rupture and heart attacks, demonstrate high levels of serum CD40L, raising prospects of targeting the TNF/TNFR SFPs in human atherosclerosis (Aukrust et al., 1999).

#### Regulators of Bone and Mammary Gland Homeostasis

Besides lacking secondary lymph nodes, mice deficient in RANKL or its receptor, RANK, have severe osteopetrosis (increased bone density) (Kong et al., 1999a). Normal skeletal homeostasis precisely balances bone-forming osteoblasts and bone-resorbing osteoclasts, derived from distinct mesenchymal and hematopoietic lineages, respectively. Bone-resorbing agents, including vitamin D<sub>3</sub>, parathyroid hormone, and proinflammatory cytokines, such as IL-1 and TNF, induce RANKL in stromal cells/osteoblasts. RANKL mediates the differentiation and activation of osteoclasts from a monocyte precursor. RANKL or RANK deficiency ablates osteoclasts, revealing an essential role in normal osteoclast survival/differentiation (Kong et al., 1999b; Kim et al., 2000b; Li et al., 2000). TRAF6-deficient mice also lack osteoclasts, indicating that this adaptor transduces RANK signals (Lomaga et al., 1999). These mutant mice have dense bones and fail to thrive due to loss of tooth eruption, a process that requires bone resorption to allow the passage of teeth through the jawbone. Hypercalcemia induced by bone-resorbing agents such as vitamin D<sub>3</sub>, parathyroid hormone, and IL-1 is defective in RANK/RANKL-deficient mice (Li et al., 2000). TNF both synergizes with RANKL to promote osteoclastogenesis (Lam et al., 2000), and acts independently to induce osteoclast development and bone resorption restricted to localized sites of injection in RANK/RANKL-deficient mice (Li et al., 2000). In infectious diseases, RANKL and TNF expressed by activated CD4<sup>+</sup> T cells can cause bone erosion (Kong et al., 1999b; Teng et al., 2000). Negative regulation of bone resorption during inflammation may be provided by IFN- $\gamma$ , which activates TRAF6 degradation, thereby blocking RANK signaling (Takayanagi et al., 2000). Thus, in both mice and humans, TNF/TNFR SFPs have central roles in regulating osteoclast differentiation and activation—and, hence, calcium storage and mobilization.

Osteoporosis—bone thinning—is a major medical problem for postmenopausal women and may be regulated by the production of OPG, a soluble RANKL decoy receptor, made by stromal cells and osteoblasts. Estrogen induces OPG, potentially explaining postmenopausal osteoporosis and the protective effects of estrogens on bone. Estrogen deficiency, at least in experimental animals, is also associated with enhanced RANKL and TNF production by T cells (Cenci et al., 2000).

In addition to their critical function in bone homeostasis, RANKL/RANK signals also govern the terminal differentiation of mammary gland alveolar buds to create lobulo-alveolar structures competent for lactation (Fata et al., 2000). Pregnant RANKL-deficient mice fail to form lactating breast tissues or produce the major milk pro-



tein,  $\beta$ -casein. Without RANK, mammary epithelial precursors undergo accelerated apoptosis because of a failure to activate the antiapoptotic Protein Kinase B (PKB/AKT). FasL/Fas may also play a role in early involution of the mammary gland postpartum (Song et al., 2000). RANKL/RANK involvement in mammary gland development during pregnancy presumably occurred with the appearance of mammals 200 million years ago and may have coordinated the transfer of minerals mobilized from the mother's bone through the milk to the growing skeleton of the newborn. Both bone metabolism and mammary gland maturation illustrate the powerful capabilities of TNF/TNFR SFPs—homeostatic regulation, spatial organization of tissues and inherently reversible effects.

#### Hair Follicle and Sweat Gland Development

Hair follicle formation in the mouse begins around E16.5–18.5 with intense NGFR expression in the mesenchymal condensation that forms the dermal papillae (Botchkareva et al., 1999). The epidermal–dermal sandwich in skin represents a complex interplay between mesenchymal dermal fibroblasts and epidermal keratinocytes. Cell renewal in hairy skin, especially re-epithelialization after injury, may involve stem cells that arise from a specialized structure, the follicular bulge, in the hair follicle (Taylor et al., 2000). The sebaceous glands develop as an appendage of the hair follicle adjacent to the bulge. During hair follicle development, NGFR expression is attenuated as the ectoderm placodes invaginate at dermal papillae to localize developing follicles. Thereafter, NGFR remains localized to the sensory neurons that innervate the follicular bulge and to cells of the outer root sheath. NGFR-deficient mice demonstrate developmentally accelerated hair follicle development, suggesting a negative regulatory effect important in temporally coordinating mesenchymal–ectodermal events (Botchkareva et al., 1999).

Additional members of the TNF/TNFR SFP—the receptors EDAR, XEDAR, and Troy, as well as the ligand EDA—help form the hair follicle. EDAR and Troy appear in developing and invaginating placodes that originate above the NGFR-rich dermal condensations. By contrast, EDA is expressed ubiquitously in developing skin in cleaved, diffusible form. Thus, spatiotemporal regulation is maintained by receptor expression. Mice deficient in either EDA (tabby) or EDAR (downless) have no primary hair follicles or sweat glands and have malformed teeth (Headon and Overbeek, 1999). Human mutations in EDA and EDAR cause similar effects (Monreal et al., 1999). The Troy locus maps near the mouse *Wv* (wavy) mutation, an autosomal dominant hair phenotype that is lethal when homozygous, hinting that Troy may be responsible for this phenotype (Kojima et al., 2000). Finally, crinkled is a mouse X-linked mutation that phenocopies EDA/EDAR deficiency, and might represent an XEDAR mutation.

#### An Unusual Regulator of Neural Development

NGFR is the most divergent member of the TNFR family, with no known TNF ligand and a propensity to dimerize rather than trimerize. NGFR shares cytoplasmic death domain-like sequences (Figure 4) and TRAF binding mo-

tifs, and supports the development of sensory neurons by interactions with high-affinity nerve growth factor receptors (Bibel and Barde, 2000). Alone, NGFR can mediate apoptosis. NGFR can associate physically with high-affinity receptors of the Trk family, however, and promote differentiation and survival, presumably reflecting differential displacement of downstream adaptors in the presence or absence of the associating receptors (Salehi et al., 2000). NGFR is critical in mediating the structural plasticity required for development of a sensory cutaneous network responsive to environmental stimuli; NGFR-deficient mice manifest severe cutaneous sensorineural defects (Lee et al., 1992).

#### Genomic and Sequence Relationships of the TNF/TNFR SFPs

In Figure 5, we show the proteins of the TNF/TNFR SF and their known ligand–receptor interactions. The proteins have been grouped according to sequence comparisons and based on the locations of their genes in the human genome. The groupings represented in the figure suggest relationships that are interesting from an evolutionary standpoint. As the genomes of different species are sequenced, it seems likely that some of these relationships will be solidified such that it may eventually be possible to trace the evolution of the SF. Although speculation along these lines is largely beyond the scope of this review, a few observations deserve brief comment.

Death domains are present in two of the ten proteins located in the clusters on human chromosomes 1 and 12. These clusters include genes that regulate the development and organization of lymphoid tissue (the genes for LT and TNF receptors) and other immunoregulatory genes, including *4-1BB*, *CD27* and *OX40* (Table 1). It seems improbable that the single exon encoding the DD was introduced separately into similar locations within single TNFR-like genes in each cluster. Instead, it is perhaps more likely that the ten genes were derived from a common ancestor that encoded a TNFR-like receptor linked to a cytoplasmic DD. If so, the recent expansion of the TNFR SF apparently favored the replication of genes encoding receptors that could signal not through direct linkage to a DD, but instead by interactions with other adaptors (most notably TRAFs). TRAFs require only a short stretch of amino acids for binding, a characteristic that could allow for rapid functional diversification during gene duplication and divergence (McWhirter et al., 1999; Park et al., 1999; Ye et al., 1999). The genomic organization thus hints at a key role for DD-containing receptors in the evolution of the modern immune system, with the later addition of derivative receptors for more specialized functions.

Troy, EDAR, and XEDAR share protein sequence (Figure 3) and functional similarities, although here again, only EDAR has a DD-related sequence in its cytoplasmic region. EDAR and XEDAR bind to the products of the EDA-A1 and EDA-A2 transcripts, which are alternatively spliced RNAs derived from the X-linked *EDA* gene (Yan et al., 2000b). Striking patches of identity in the DDs of EDAR and NGFR (Figure 4) raise the possibility that EDAR could have diverged from the NGFR gene and then subsequently given rise to Troy and XEDAR. As



mentioned above, an early founding role for the NGFR in the evolution of the TNFR SF might also be suggested by the fact that it does not bind a TNF-related ligand, and by the fact that (like the *DR6* and *OPG* genes) its gene lacks the typical distribution of introns in its CRD-encoding region (Figure 5). Interestingly, *TACI* and *BCMA* share loose protein sequence similarity with *Troy*, *EDAR*, and *XEDAR* (Figure 3) and the genes encoding their ligands (*APRIL* and *BLYS*) have introns placed in similar locations to those in *EDA*. From such observations, it is possible to suggest that the genes for *TACI* and *BCMA* may have derived from the *NGFR* gene through duplication of an intermediate *EDAR*-related gene. As with the genes on chromosomes 1 and 12, if this hypothesis is accurate, the expansion of the TNFR SF has involved the generation of additional specialized receptors from founder DD-containing receptors.

#### Mechanisms of Genetic Diseases of TNFRs

There are four well-defined genetic diseases that affect ligand:receptor interactions in the TNFR SF: X-linked hyper IgM syndrome (HIGM-1, involving CD40:CD40L), the autoimmune lymphoproliferative syndrome (ALPS, involving Fas:FasL), TNF receptor-associated periodic syndrome (TRAPS, involving TNFR1:TNF), and anhidrotic ectodermal dysplasia (EDA, involving *EDAR*/*XEDAR*:*EDA*). A fifth disease, familial expansile osteolysis (FEO, involving *RANK*/*RANKL*), has been linked with signal peptide mutations in *RANK* and leads to lytic bone lesions (Hughes et al., 2000). The most common genetic mechanism for most cases of ALPS is autosomal dominance (gain of function), involving heterozygous dominant-interfering alleles. These alleles encode defective Fas proteins that complex with normal Fas proteins, thereby impairing apoptosis signaling and causing marked lymphoid expansion and autoimmunity (Lenardo et al., 1999; Siegel et al., 2000). Afflicted individuals develop pathogenic auto-antibodies—frequently against hematopoietic cells—that cause hemolytic anemia, thrombocytopenia, or neutropenia (Rieux-Laucat et al., 1995). In TRAPS, heterozygous dominant alleles of *TNFR1* with amino acid changes in the extracellular domain apparently cause enhancement of the proinflammatory effects of TNF. This may result, at least in part, from a decrease in *TNFR1* shedding (Galon et al., 2000). By contrast, HIGM-1 is typically caused by recessive mutations (loss of function) in the X-linked gene encoding CD40L. Hence, this severe immunodeficiency, characterized by defective CD40-mediated antibody class switching in B cells, is found mostly in males and rarely in females with skewed X-chromosome inactivation (Notarangelo and Hayward, 2000). EDA can result from either dominant or recessive mutations, but in both cases, the genetic aberrations cause defective ligand-receptor interactions necessary for the normal differentiation of ectodermal placodes into hair, sweat glands, and teeth (Yan et al., 2000b). FEO, as well as some cases of familial Paget disease of bone (PDB), has been linked with signal peptide mutations in *RANK* that lead to activating phenotypes (Hughes et al., 2000). Dominant inheritance results in the appearance in young adulthood of expansile osteolytic lesions in the long bones (FEO) or skull and pelvis (PDB). Early deafness and loss of adult teeth due

to resorption are common clinical manifestations. Together, these diseases underscore the diverse functions mediated by TNF/TNFR SFP and illustrate the types of genetic mechanisms that could underlie diseases involving other TNF/TNFR SFPs.

#### Conclusions: Looking to the Past

We have attempted to summarize and conceptualize the structure, function, and genomic organization of the TNF/TNFR SFP. Functional studies based on gene knockout mice and natural human mutations support a role for these proteins in modulating dynamic, multicellular interactions important to diverse developmental and homeostatic needs. The pairing of modular cysteine-rich ectodomains with existing internal death domains and the evolution of TRAF adaptor binding motifs provided a powerful signaling mechanism used repeatedly in different contexts for coupling diverse environmental stimuli to downstream differentiation pathways. These downstream adaptor/signaling modules were previously tested in evolution, with members represented in the genomes of flies and worms that had already been adapted to body plan development and innate immunity. Although no clear TNFR-like homologs have been discovered, FADD-, RIP-, and TRAF-like molecules have been identified in *Drosophila* (Khush and Lemaitre, 2000), and TRAF-like molecules in *C. elegans* (Wajant et al., 1999). Caspases are present in both organisms (Ruvkun and Hobert, 1998; Rubin et al., 2000). Recently, the gene for a death receptor-like molecule was identified in zebrafish (Long et al., 2000). Further studies in birds and fish will be required to trace the process by which TNF/TNFR SFP became peculiarly adapted to mammals. NGFR, like other neurotrophins, is found only in vertebrates, with the earliest genes identified in jawless fish, which arose about 460 million years ago during the period of acquisition of the RAG genes critical for the subsequent development of adaptive immunity (Hallbook, 1999). A TNF homolog in bony (teleost) fish suggests divergence from mammalian TNF long before the appearance of LT—found thus far only in mammals and marsupials—but more extensive comparative analyses will be necessary (Hirono et al., 2000). Intriguingly, the 3-fold jelly-roll structure of TNF is remarkably similar to the capsid proteins of small RNA viruses (Fesik, 2000). Several avian virus receptors are TNFRs, and human HveA supports herpes virus attachment (Mauri et al., 1998; Adkins et al., 2000). The similar intracellular signaling domains of some TNFR and TLRs, which function as germline-encoded receptors for pathogen-encoded structures, raise the possibility that TNF ligands represent descendents from the horizontal capture of a gene encoded by an ancient viral pathogen.

If, as argued above, TNF/TNFR SFP gene diversification radiated from early NGFR-like or *TNFR1*-like precursors, evolution has apparently found these molecules to be valuable instruments in generating multicellular organs and transient microenvironments, or neotissues, that respond to external environmental conditions. These multicellular responses have evolved to be rapid, reversible and ultimately, to achieve equipoise in cellular numbers and activation. To achieve this, signaling via TNF/TNFR SFPs is spatially constrained during the orga-

nization of neo-tissues and then dispersed upon removal of tonic signals. As we hope to have illustrated, these functions have been most elaborately explored in the immune system, but also govern similar adaptive responses in bone, skin, mammary gland, and perhaps other organ systems whose molecular organization awaits discovery.

#### Acknowledgments

The authors regret the inability to cite all of the primary literature contributing to this review due to length considerations. The authors thank F. Chan, T. Migone, M. Peter, J. Puck, R. Siegel, H. Walczak, and J. Wang for insightful comments on the manuscript. N. K. is a Scholar of the Leukemia Society. Supported in part by grants from the National Institutes of Health (R. M. L., N. K.).

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# Tumor Necrosis Factor: An Apoptosis JuNKie?

## Review

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TNF's main function is to stimulate inflammation by turning on gene transcription through the IKK/NF- $\kappa$ B and JNK/AP-1 signaling cascades. TNF also can trigger apoptosis through caspase-8, but the role and underlying mechanism of this activity are not fully understood. Here, we review recent data on the role of JNK in the regulation of TNF-dependent apoptosis and discuss what is known so far about how cells decide whether to live or die in response to TNF.

### TNF as a Key Inducer of Proinflammatory Genes

Tumor necrosis factor- $\alpha$  (TNF) plays a pivotal role in orchestrating innate inflammatory responses in vertebrates. Upon detection of invading intracellular pathogens, tissue macrophages and T cells produce either membrane-associated TNF (mTNF) or proteolytically derived soluble TNF (sTNF). TNF triggers local expression of chemokines and cytokines, promoting the adhesion, extravasation, attraction, and activation of leukocytes at the site of infection. Later, TNF facilitates transition from innate to acquired immunity by enhancing antigen presentation and T cell costimulation.

TNF is the prototype of ~20 related cytokines that act through specific members of the TNF receptor (TNFR) superfamily, mainly to modulate immunity (reviewed in Locksley et al., 2001). TNF homologs exist in insects, primitive chordates, amphibians, fish, birds, and mammals. Mammalian TNF signals through two distinct cell-surface receptors: TNFR1, the primary receptor for sTNF, and TNFR2, the main receptor for mTNF (reviewed in Wajant et al., 2003). These receptors trigger several intracellular signaling pathways, including the I- $\kappa$ B kinase (IKK), c-Jun N-terminal kinase (JNK), and p38 or p42/44 mitogen-activated protein kinase (MAPK) cascades, which control gene expression through transcription factors such as NF- $\kappa$ B and AP-1.

Most cell types constitutively express TNFR1 while TNFR2 expression is highly regulated. TNFR1 and 2 resemble each other in their extracellular, cysteine-rich domains. TNFR1 contains a cytoplasmic death domain (DD) that binds to the adaptor TRADD (TNFR-associated DD). TNFR2 lacks a DD, but has a cytoplasmic motif that binds TRAFs (TNFR-associated factors). The homotrimeric TNF ligand binds a pre-associated receptor homotrimer, inducing conformational changes that enable the cytoplasmic motifs to bind cognate signaling adaptors (Locksley et al., 2001). Upon binding to ligated TNFR1, TRADD recruits the secondary adaptors RIP1, TRAF2, or TRAF5. This causes activation of the IKK complex, which consists of IKK  $\alpha$ ,  $\beta$ , and  $\gamma$  (also called Nemo), through an unknown mechanism (reviewed in

Chen and Goeddel, 2002) (Figure 1). IKK stimulates NF- $\kappa$ B by catalyzing phosphorylation and degradation of the NF- $\kappa$ B inhibitor, I- $\kappa$ B (reviewed in Karin and Lin, 2002). TRAF2 functions also as an obligatory conduit for stimulation of JNK through its MAPK kinase MKK7, promoting phosphorylation of c-Jun and thus increasing AP-1 activity. Unlike TNFR1, TNFR2 binds TRAF2 directly, hence activating IKK and JNK (Figure 1). TRAF2 also recruits ancillary proteins that modulate signaling through each TNFR, i.e., cIAP (cellular inhibitor of apoptosis protein) 1 and 2, and TRAF1. cIAP1 supports ubiquitination and degradation of TRAF2, whereas TRAF1 inhibits TNFR2-dependent signaling through an unknown mechanism (Wajant et al., 2003).

### TNF as a Conditional Death Ligand Blocked by NF- $\kappa$ B

While TNF's cardinal role is to stimulate inflammation, it is capable also of inducing apoptosis when NF- $\kappa$ B signaling is blocked. The precise biological role of this activity is unclear. TNF may function alongside of "professional" death ligands such as FasL and Apo2L/TRAIL to help cytotoxic leukocytes kill pathogen-infected cells. Perhaps TNF's apoptotic capability contributes to its established pathological role in rheumatoid arthritis and inflammatory bowel disease.

The "intrinsic" apoptosis pathway—triggered by intracellular injury such as DNA damage—controls caspase activation through the Bcl-2 gene family (reviewed in Puthalakath and Strasser, 2002; Cory and Adams, 2002). In this pathway, damage sensors induce transcription of Bcl-2-homology 3 (BH3) domain proteins (e.g., Puma, Noxa, Bim, Bmf). These apical regulators activate downstream proapoptotic Bcl-2 relatives (e.g., Bax, Bak), overcoming inhibition by antiapoptotic Bcl-2 family members (e.g., Bcl-2, Bcl-X<sub>L</sub>). The activated Bcl-2 relatives trigger mitochondrial release of factors that promote caspase activation in the cytosol. One factor is cytochrome c, which cooperates with Apaf-1 to activate caspase-9. This apical caspase activates the effector caspases 3, 6, and 7, causing apoptotic death. Two other mitochondrial factors, Smac/Diablo and Omi/HtrA2, prevent IAPs from inhibiting caspase activation.

The "extrinsic" pathway is triggered by extracellular death ligands such as the TNF relatives FasL and Apo2L/TRAIL, which signal respectively through Fas and DR4 or DR5 (reviewed in Ashkenazi, 2002). These death receptors bind directly to the adaptor FADD (Fas-associated DD), which mediates recruitment and activation of caspases-8 and -10 within a death-inducing signaling complex (DISC). Caspases-8 and -10 activate apoptotic death through the same effector caspases as the intrinsic pathway. Modulation of the extrinsic pathway occurs at several levels. Decoy receptors can compete with death receptors for ligand binding. The caspase-related molecule c-FLIP (cellular FLICE-inhibitory protein), which lacks catalytic activity, competes with caspases-8 and -10 for DISC binding (reviewed in Thome and Tschopp, 2001). Further downstream, IAPs inhibit

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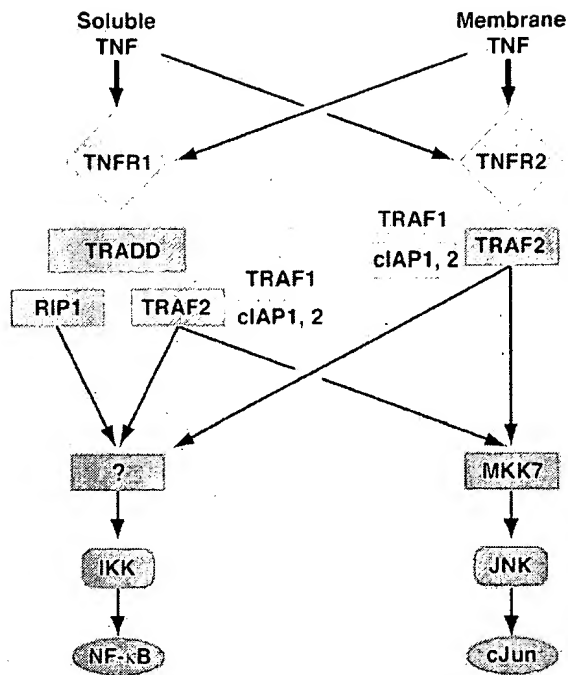


Figure 1. Model for Control of Gene Transcription by TNF

effector caspase activation (reviewed in Salvesen and Duckett, 2002). In "type I cells," the DISC generates sufficient caspase activity to trigger death. In "type II cells," apical caspase activation is weaker, and apoptosis requires amplification through crosstalk to the intrinsic pathway: caspase-8 cleaves the BH3 protein Bid, which stimulates Bax and Bak to augment caspase activation (reviewed in Peter and Krammer, 2003).

The pleiotropic nature of TNF has hindered elucidation of its apoptosis signaling mechanism. TNF does not usually trigger apoptosis in TNFR-bearing cells. However, general inhibition of transcription or translation or selective blockade of the IKK/NF- $\kappa$ B pathway uncovers TNF's proapoptotic capacity (Chen and Goeddel, 2002; Karin and Lin, 2002). Although mice with *TNF* or *TNFR* gene knockouts develop normally, mice deficient in NF- $\kappa$ B signaling die in utero from TNF-dependent apoptosis of liver cells. By activating NF- $\kappa$ B, TNF induces a number of antiapoptotic genes, including *c-FLIP*, *cIAP1*, *cIAP2*, *A1*, *A20*, *TRAF1*, and *TRAF2*.

#### TNFR1 and 2 as Mediators of TNF-Induced Apoptosis

Challenge of mice with bacterial lipopolysaccharide (LPS) together with the liver-specific transcription inhibitor D-galactosamine (GalN) stimulates systemic release of sTNF, which induces hepatocyte apoptosis and liver failure. TNFR1-deficient mice are resistant to this effect, while TNFR2 knockouts are sensitive. Thus, sTNF signals hepatocyte apoptosis mainly through TNFR1. Indeed, TNF-deficient mice expressing a transgenic, non-cleavable mTNF mutant are largely resistant to LPS/GalN challenge. Unlike LPS, the T cell stimulator Concanavalin A (ConA) induces mostly mTNF. ConA causes

liver damage that requires both TNFR1 and 2 for maximal apoptosis. Thus, mTNF uses both receptors to trigger hepatocyte death (Maeda et al., 2003 and references therein).

TNFR1 shares several components of the extrinsic death pathway with Fas, DR4, and DR5. Mouse embryo fibroblasts (MEFs) deficient in either FADD or caspase-8 resist TNF-induced apoptosis, demonstrating an obligatory role for these molecules. Previous studies with transfected cells suggested that TNFR1 assembles a DISC similar to that of Fas and DR4/5, except that this occurs indirectly through TRADD (Chen and Goeddel, 2002). A recent study examined a wild-type HT1080 human fibroblast cell line, resistant to sTNF-induced apoptosis, and a mutant line with defective NF- $\kappa$ B activation, sensitive to TNF killing (Micheau and Tschopp, 2003). In both lines, sTNF induces TRADD-mediated assembly of a TNFR1-associated complex (complex I) that contains RIP1, TRAF2, and cIAP1 and activates the IKK/NF- $\kappa$ B pathway (Figure 2). Subsequently, TRADD, RIP1, and TRAF2 undergo biochemical modifications and the complex dissociates from TNFR1, moving to the cytosol. FADD and caspase-8 bind to this cytosolic complex (complex II). In wild-type cells, complex II contains abundant c-FLIP but little caspase-10, while in mutant cells the converse is true. Thus, c-FLIP may be an important NF- $\kappa$ B-dependent factor preventing apical caspase activation at the level of complex II, possibly by competing for caspase-10 binding. Besides c-FLIP, cIAP1 and TRAF1 are more abundant in complex II of the mutant cells and might inhibit caspase activation (Micheau and Tschopp, 2003; Wang et al., 1998).

Simultaneous engagement of both TNFRs amplifies TNF-induced apoptosis (Wajant et al., 2003; Maeda et al., 2003). This correlates with increased TNFR2-induced TRAF2 degradation. Since TRAF2 recruits cIAPs to TNFR1, its degradation via TNFR2 may facilitate cell death. TRAF2 destruction also attenuates TNFR1-mediated NF- $\kappa$ B activation, further promoting apoptosis. Thus, TRAF2 may provide an additional switch between inflammation and cell death downstream of TNF.

#### JNK as a Regulator of Apoptosis

JNK1, 2, and 3 (also known as stress-activated protein kinases) form a subgroup of the MAPK superfamily that is activated by cell stressors such as ultraviolet (UV) radiation and by proinflammatory cytokines such as TNF and interleukin-1 (reviewed in Weston and Davis, 2002; Shaulian and Karin, 2002; Lin, 2003). JNK phosphorylates specific subunits, namely c-Jun, JunB, JunD, and ATF-2, of the AP-1 transcription factor, turning on genes that control diverse cellular functions including proliferation, differentiation, and apoptosis. JNK's precise role in apoptosis remains controversial since it appears to have conflicting effects depending on the species, type of cell, or nature of death stimulus.

*Drosophila melanogaster* has counterparts to the major components of the mammalian JNK cascade as well as orthologs of many mammalian cell death genes. In *Drosophila*, apoptosis during embryonic patterning of the wing, eye, and gut requires the fly's JNK ortholog *DJNK* (also called *Basket*) (Kockel et al., 2001; Moreno et al., 2002; and references therein) (Figure 3). Ectopic

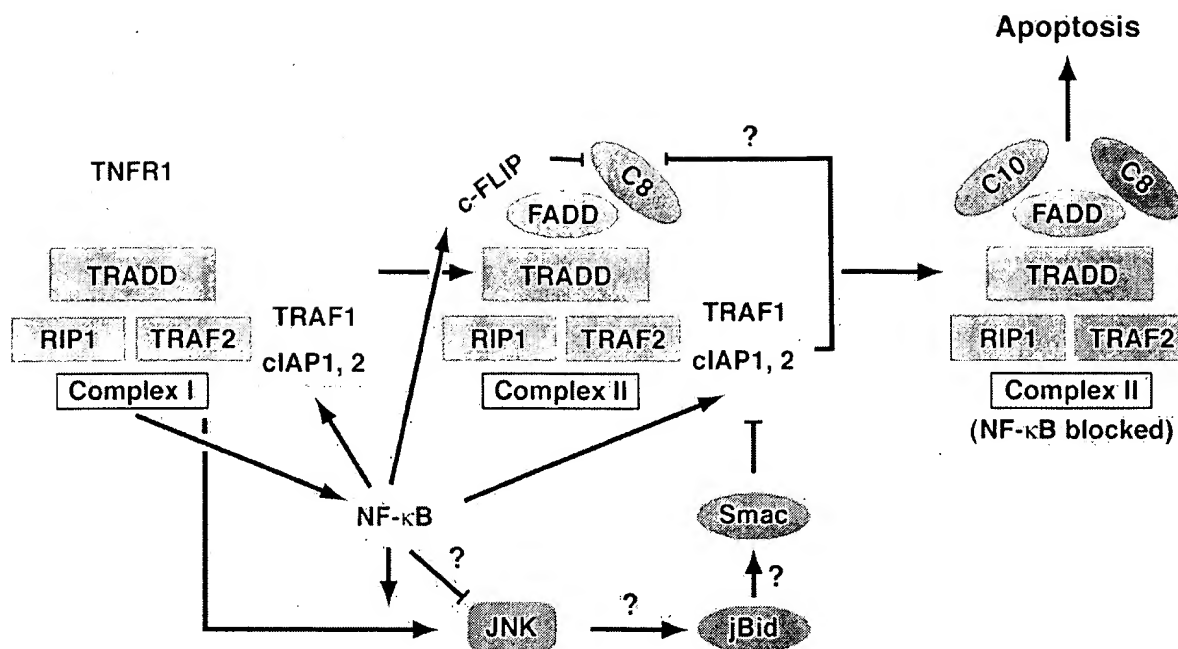


Figure 2. Model for Apoptosis Control by TNFR1

DJNK activation in the eye imaginal disc causes excessive cell death and eye ablation. This phenotype involves DJNK-dependent phosphorylation of DJun, which promotes transcription of *Hid* and *Rpr*. *Hid*, *Rpr*, and another fly gene called *Grim* encode proteins with a related sequence motif that enables them to induce cell death by binding to the fly IAP ortholog DIAP1. This binding prevents DIAP1 from blocking activation of the fly caspase DRONC, much like the interaction of mammalian Smac and IAPs (Salvesen and Duckett, 2002). DIAP1 also attenuates DJNK activation by promoting degradation of DTRAF1, the fly ortholog of mammalian TRAF2. DJun turns on an additional negative-regulatory feedback loop by promoting transcription of *Puckered* (*Puc*), a dual specificity phosphatase that inactivates DJNK. Thus, in *Drosophila*, JNK plays a crucial role in a tightly regulated signaling pathway that promotes apoptosis.

In mammals, there is evidence both for proapoptotic and for antiapoptotic JNK activity (Lin, 2003 and references therein). Apoptotic death of rat PC12 neuronal cells deprived of nerve growth factor (NGF) requires JNK activity. Knockout of *JNK1* and *JNK2* in the mouse suppresses apoptosis in the hindbrain neuroepithelium at day 9.25, but causes increased apoptosis in the hindbrain and forebrain at day 10.5. *JNK1/JNK2*-deficient MEFs resist apoptosis induction by UV radiation, proteasome inhibitors, or genotoxic drugs. Furthermore, in *JNK1* or *JNK2* knockout mice, thymocytes are refractory to death in response to T cell receptor ligation, while in *JNK3*-deficient mice, hippocampal neurons resist apoptosis induction by excitotoxic stress. JNK may promote mammalian cell apoptosis by engaging the cell-intrinsic pathway (Figure 4). Whereas wild-type MEFs die in response to UV or as a result of ectopic expression of a

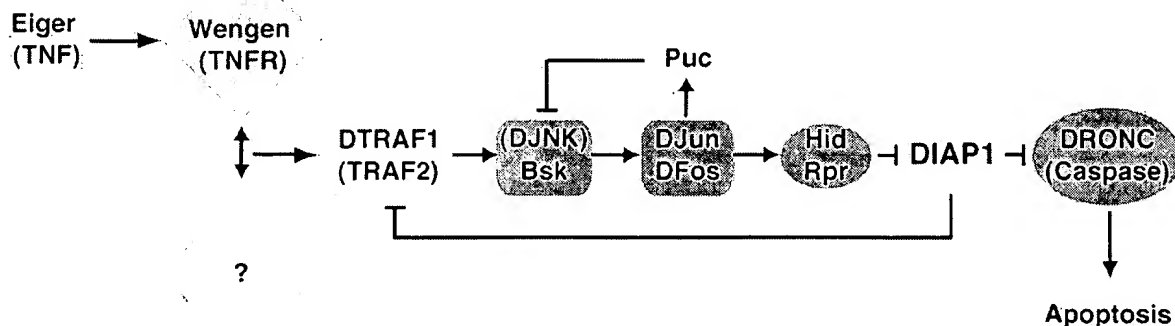


Figure 3. Model for Apoptosis Control by *Drosophila* Eiger



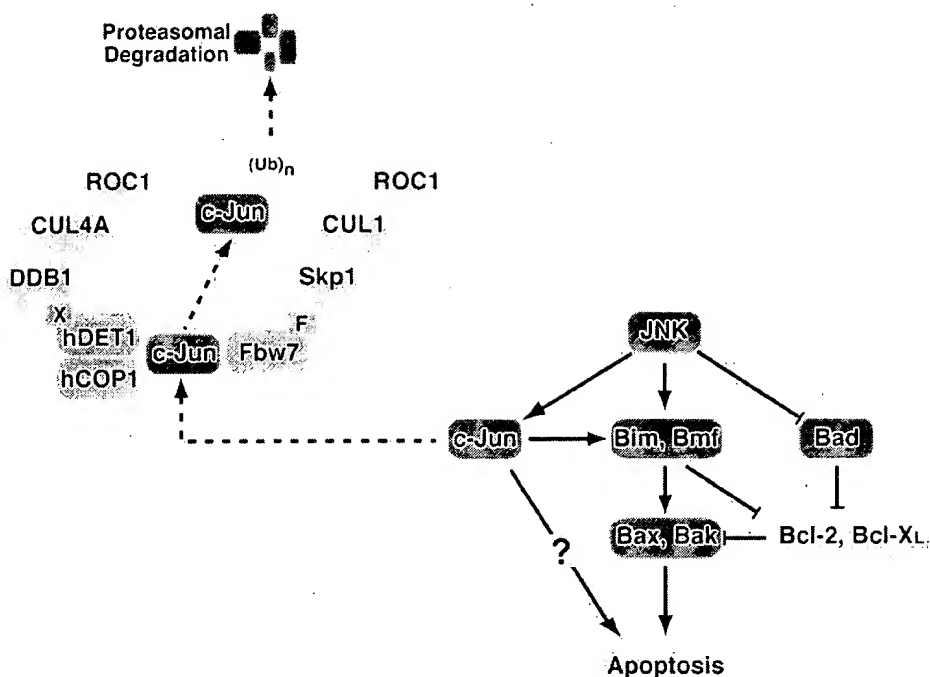


Figure 4. Model for Mammalian Apoptosis Modulation by JNK

constitutively active MKK7-JNK fusion protein, *Bax/Bak* knockout MEFs do not (Lei et al., 2002). Moreover, *JNK1/JNK2*-deficient MEFs fail to show *Bax* activation, cytochrome *c* release, or death upon UV exposure. JNK-dependent phosphorylation of the BH3 proteins *Bim* and *Bmf* causes their dissociation from dynein and myosin motor complexes, as does UV radiation; this may free *Bim* and *Bmf* to activate apoptosis through *Bax* and *Bak* (Lei and Davis 2003 and references therein). JNK also promotes *Bim* transcription through *c-Jun* (Shaulian and Karin, 2002).

In contrast to the proapoptotic activity of JNK in NGF-deprived PC12 cells, new data suggest that JNK mediates pro-survival signals downstream of interleukin-3 (IL-3) in human FL5.12 pro-B cells (Yu et al., 2004). IL-3, a crucial survival factor for FL5.12 cells, stimulates JNK, while its withdrawal decreases JNK activity. Inhibition of JNK with the low molecular weight compound SP600125 partially attenuates apoptosis of FL5.12 cells after IL-3 withdrawal, whereas expression of a constitutively active *JNKK2-JNK1* fusion protein promotes this response. Thus, JNK contributes to survival signaling in FL5.12 cells downstream of IL-3. Earlier work showed that IL-3 promotes phosphorylation of the BH3 protein *Bad* predominantly on serine residues (Ser122 and 136), and that this phosphorylation mediates the survival signal of IL-3 in FL5.12 cells; current work shows that activated JNK phosphorylates *Bad* on threonine 201; this inhibits *Bad*'s association with *Bcl-X<sub>L</sub>*, probably freeing up more *Bcl-X<sub>L</sub>* to block apoptosis (Yu et al., 2004 and references therein). These findings suggest a novel mechanism for JNK-dependent inhibition of cell death (Figure 4). However, the prevalence of *Bad* phosphorylation on threonine 201 by JNK, as compared to phosphorylation on serines by other kinases that may promote

survival downstream of IL-3, e.g., *Akt*, remains to be determined.

#### JNK as a Contextual Modulator of TNF-Induced Apoptosis

While there is experimental evidence that JNK can inhibit apoptosis induction by TNF, other data suggest that it can function as an important positive regulator of TNF-induced apoptosis. In *Drosophila*, a homolog of the mammalian TNF superfamily called *Eiger* stimulates apoptosis through a JNK-dependent mechanism (Igaki et al., 2002; Moreno et al., 2002; and references therein) (Figure 3). *Eiger* binds to a TNFR-related fly protein called *Wengen* (Kanda et al., 2002). Although *Wengen* is required for *Eiger* activity, it does not possess recognizable binding motifs for signaling adaptors such as TRAFs or TRADD, nor does it display detectable signaling function. Hence, *Wengen* may be a ligand binding subunit of a more complex signaling receptor. Regardless, *Eiger* acts through *Wengen* to induce DTRAF1-dependent stimulation of *DJNK*, thereby inducing apoptosis. As with *DJNK*, ectopic *Eiger* expression in the eye results in excessive apoptosis and eye ablation, and this phenotype can be suppressed by *Puc*.

Several studies in mice demonstrate an inhibitory role for JNK in TNF-induced cell death. *TRAF2* knockout MEFs, which have largely intact NF- $\kappa$ B signaling but reduced JNK stimulation, display increased apoptosis sensitivity to TNF (Wajant et al., 2003). The JNK inhibitor SP600125 enhances TNF-induced apoptosis of MEFs deficient in the *RelA* subunit of NF- $\kappa$ B. Furthermore, MEFs deficient in *JNK1* and *JNK2* show increased sensitivity to TNF killing, and their transfection with *JNK1* or *JunD* rescues TNF resistance (Lamb et al., 2003 and references therein). Conversely, *JNK2*-deficient MEFs,

isolated from a different mouse genetic background than the latter MEFs, display a moderate resistance rather than sensitization to TNF killing (Dietrich et al., 2003). Moreover, constitutive JNK1/2 activation in cells with deficient NF- $\kappa$ B signaling sensitizes to TNF-induced apoptosis (Lin, 2003). *JNK1* and *JNK2* knockouts are much less sensitive than wild-type mice to ConA-induced liver damage (which involves mTNF); furthermore, SP600125 blocks TNF-induced death of *IKK $\beta$* -deficient mouse hepatocytes (Maeda et al., 2003).

JNK's involvement in TNF-induced apoptosis may depend on NF- $\kappa$ B. TNF activates JNK transiently; however, in cells with general inhibition of transcription or specific inhibition of NF- $\kappa$ B, TNF leads instead to prolonged JNK activation (Lin, 2003; Maeda et al., 2003). The NF- $\kappa$ B-dependent genes *A20*, *GADD45 $\beta$* , and *XIAP* attenuate TNF activation of JNK upon ectopic expression (Lin, 2003). However, *A20*-deficient MEFs, which display increased sensitivity to TNF-induced death, have normal JNK activation (Lee et al., 2000). Similarly, *GADD45 $\beta$* -deficient MEFs or mouse splenocytes show unaltered JNK activation or apoptosis induction by TNF plus cycloheximide, although this result has been challenged (Amanullah et al., 2003). While *XIAP* knockout mice develop normally (Harlin et al., 2001), sensitivity of *XIAP*-deficient cells to TNF stimulation of JNK or apoptosis has yet to be analyzed. Thus, the specific mechanisms by which NF- $\kappa$ B limits the duration of JNK activation remain obscure.

A recent study suggests that JNK may play an essential positive role in TNF-induced apoptosis (Deng et al., 2003). In *RelA*-deficient MEFs or human HeLa cells expressing an *I- $\kappa$ B* mutant that blocks NF- $\kappa$ B, small interfering RNA (siRNA) knockdown of MKK7 prevented TNF-induced caspase-8 processing and cell death. TNF stimulated an MKK7-dependent (and presumably, JNK-dependent) cleavage of the BH3 protein Bid. MKK7/JNK-mediated Bid processing generated a unique product (termed jBid), distinct from the previously identified product of Bid cleavage by caspase-8. A Bid deletion mutant similar to jBid in size translocated to the mitochondria, where it selectively triggered the release of Smac, but not of cytochrome c. siRNA knockdown of Smac prevented TNF from inducing caspase-8 processing and apoptosis, while transfection of activated Smac augmented these TNF effects, suggesting that Smac acts upstream of caspase-8 activation. A Smac-based peptide, known from other studies to block IAP interactions with caspases, inhibited association of cIAP1 and TRAF2. These data suggest a model in which TNF activates caspase-8 by relieving it from cIAP inhibition through MKK7/JNK-dependent jBid generation and consequent Smac release (Figure 2). However, despite evidence for inhibitory association of cIAPs with caspases-3, -7, or -9, support for interaction with caspase-8 (or 10) is lacking (Salvesen and Puckett, 2002). Moreover, overexpression of cIAP1 and 2 in HT1080 cells expressing mutant *I- $\kappa$ B* did not protect against TNF killing (Wajant et al., 2003). So it remains unclear how jBid promotes caspase-8 activation through Smac. The jBid model raises several other intriguing questions: (1) How does MKK7/JNK activation generate jBid? Is transcription required? (2) Do inhibitors of caspases or other proteases block jBid generation? (3) What is jBid's

N-terminal structure? (4) How does jBid selectively release mitochondrial Smac without affecting cytochrome c? (5) Does ectopic expression of jBid inhibit the association of cIAP1 with TRAF2 within complex II? (6) Is jBid involved in TNF-induced apoptosis in type I as well as type II cells?

Besides acting at the level of direct or indirect post-transcriptional modification of death signaling molecules, JNK may modulate apoptosis through AP-1-dependent gene transcription. This is exemplified by JNK's regulation of Bim mRNA levels through c-Jun (Shaulian and Karin, 2002). The level of c-Jun in cells is controlled by ubiquitination and consequent proteasomal degradation, but the underlying enzymatic machinery has been elusive. New work uncovers two specific ubiquitin ligases that support c-Jun destruction in neuronal and nonneuronal cells, affecting AP-1 activity as well as apoptosis (Nateri et al., 2004; Wertz et al., 2004; and references therein) (Figure 4). Numerous mammalian F box proteins function as substrate adaptors for ubiquitin ligases of the SCF (Skp1/Cullin/F box protein) type. A yeast two-hybrid screen of a brain cDNA library, designed to identify specific binders of phosphorylated c-Jun, detected the F box protein Fbw7 (Nateri et al., 2004). Fbw7 bound to phosphorylated c-Jun and promoted its ubiquitination, but did not interact with a phosphorylation-defective c-Jun mutant or with ATF2. Ectopic expression of Fbw7 and c-Jun in 293T human embryonic kidney cells induced proteasomal degradation of phosphorylated c-Jun, attenuating AP-1 activity. Conversely, siRNA knockdown of Fbw7 in rat PC12 cells led to accumulation of phospho-c-Jun, increasing AP-1 activity. Importantly, Fbw7 depletion in PC12 cells elevated basal apoptosis levels and substantially augmented apoptosis induced by NGF deprivation. Expression of the JNK-inhibitory scaffolding protein JIP-1 reversed these proapoptotic effects, indicating that they are required for JNK activity. Fbw7 depletion also augmented apoptosis of mouse primary cerebellar neurons in a c-Jun-dependent fashion. Thus, in neuronal cells, modulation of c-Jun levels by the SCF<sup>Fbw7</sup> ubiquitin ligase complex appears to provide an important mechanism for controlling JNK-dependent apoptosis (Nateri et al., 2004).

Different ubiquitin ligase complexes may control c-Jun degradation in other tissues. Indeed, pulldown experiments with hDET1 (the human homolog of de-ETiolated 1, an *Arabidopsis thaliana* protein that regulates plant photomorphogenesis), identified a distinct ubiquitin ligase complex that regulates c-Jun ubiquitination in 293T epithelial cells and in U2OS osteosarcoma cells (Wertz et al., 2004) (Figure 4). hDET1 binds to hCOP1, a human homolog of *Arabidopsis Constitutively Photomorphogenic-1*, together forming a ubiquitin ligase substrate adaptor that brings c-Jun into contact with a ubiquitin ligase complex that consists of three proteins: DNA damage binding protein 1 (DDB1), Cullin 4A (CUL4A), and Regulator of Cullins-1 (ROC1). Like Fbw7, hCOP1 possesses WD40 repeats; however, neither hDET1 nor hCOP1 contains an F box, suggesting that an unidentified "X box" mediates binding of the hDET1/hCOP1 heterodimer to the rest of the complex (Figure 4). This ligase complex, termed DCX<sup>hDET1/hCOP1</sup>, catalyzed ubiquitination of c-Jun in vitro and in 293T

and U2OS cells. Ectopic coexpression of *hCOP1* and *hDET1* led to proteasome-mediated c-Jun degradation, and siRNA knockdown of *hDET1* led to c-Jun accumulation, increasing AP-1 activity and apoptosis. Thus, in nonneuronal cells, modulation of c-Jun levels by the DCX<sup>hDET1/hCOP1</sup> complex provides an important mechanism for controlling JNK-dependent apoptosis, similar to the action of SCF<sup>Flw7</sup> in neuronal cells. Given the extensive diversity of the ubiquitin-proteasomal system, it is likely that multiple ubiquitin ligases regulate JNK-dependent apoptosis in various cell types at the level of c-Jun and perhaps of other AP-1 subunits. These new findings raise the intriguing, as yet untested hypothesis that ubiquitin ligase activity against c-Jun may regulate JNK's contribution to TNF-induced apoptosis. If so, then a logical question that follows is whether NF- $\kappa$ B uses this mechanism to exert additional inhibitory control over TNF-induced cell death, perhaps by promoting the expression of specific ubiquitin ligase components.

### Conclusions

TNF's primary role is to stimulate inflammatory cells to fight infection, whereas the function of its proapoptotic capability remains mysterious. Nonetheless, it makes sense that the main conduit for TNF's proinflammatory action, NF- $\kappa$ B, strongly inhibits TNF's induction of apoptosis. TNF turns on several antiapoptotic genes through NF- $\kappa$ B. Notable amongst those is c-FLIP, which blocks apical caspase activation in a cytosolic DISC that forms downstream of TNFR1. NF- $\kappa$ B stimulation also limits the duration of TNF-induced JNK activity; conversely, NF- $\kappa$ B inhibition is associated with prolonged TNF-induced JNK activation and apoptosis. How NF- $\kappa$ B controls JNK activity is not yet clear, though several NF- $\kappa$ B-induced genes have been implicated.

New evidence supports a positive, if not essential, role of JNK in TNF-induced apoptosis. In *Drosophila*, the TNF superfamily homolog Eiger requires *DJNK* to induce cell death. However, Eiger engages the DRONC caspase independently of DFADD and the *Drosophila* caspase-8 homolog DREDD, which in the fly regulate inflammation rather than apoptosis. In contrast, mammalian TNF requires TRADD, FADD, and caspase-8 for apoptosis induction, and JNK may modulate this by promoting caspase-8 activation. In cells with blocked NF- $\kappa$ B, JNK may trigger an unusual path to caspase-8 activation, involving a novel modification of Bid. Future work should define biochemically how JNK supports Bid processing and caspase-8 activation.

Both in neuronal and nonneuronal cells, newly identified ubiquitin ligase complexes that support proteasomal degradation of c-Jun reveal a novel mechanism for control of JNK-associated apoptosis. It will be interesting to examine whether this mechanism also modulates JNK's contribution to TNF-induced apoptosis, and if so, whether NF- $\kappa$ B affects the transcription of specific ubiquitin ligase components. The broader question of how cells integrate NF- $\kappa$ B-controlled determinants with other signals downstream of TNF is ripe for the emerging field of systems biology. Indeed, the first example for such exploration of TNF/NF- $\kappa$ B signaling has just been reported (Bouwmeester et al., 2004). Understanding how cells choose life or death after TNF stimulation will un-

doubtedly shed new light on the role of TNF-induced apoptosis in health and disease.

### Acknowledgments

We thank Chris Clark for editing this manuscript.

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# Whole-Genome Analysis of Dorsal-Ventral Patterning in the *Drosophila* Embryo

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## Summary

The maternal Dorsal regulatory gradient initiates the differentiation of several tissues in the early *Drosophila* embryo. Whole-genome microarray assays identified as many as 40 new Dorsal target genes, which encode a broad spectrum of cell signaling proteins and transcription factors. Evidence is presented that a tissue-specific form of the NF- $\kappa$ B transcription complex is essential for the activation of gene expression in the mesoderm. Tissue-specific enhancers were identified for new Dorsal target genes, and bioinformatics methods identified conserved *cis*-regulatory elements for coordinately regulated genes that respond to similar thresholds of the Dorsal gradient. The new Dorsal target genes and enhancers represent one of the most extensive gene networks known for any developmental process.

## Introduction

Gradient morphogens control a variety of metazoan patterning processes, including the primary axes of the *Drosophila* embryo (St. Johnston and Nusslein-Volhard, 1992; Courey and Huang, 1995; Rusch and Levine, 1996), the animal cap of the *Xenopus* embryo (Gurdon and Bourillot, 2001), the vertebrate neural tube (Jessell, 2000), the *Drosophila* wing imaginal disk (Strigini and Cohen, 1999), and the limb buds of chicks and mice (Martin, 2001). In most of these examples, extracellular gradients of cell signaling molecules such as Hedgehog, BMP, and FGF trigger the formation of transcription factor gradients by the differential activation of cell surface receptors, including Patched, Thickveins/Activin, and receptor tyrosine kinases (e.g., Podos and Ferguson, 1999; Van Buskirk and Schupbach, 1999; Ingham and McMahon, 2001). The resulting regulatory gradients initiate the formation of distinct cell types through the differential regulation of target genes that implement

morphogenesis. This process is probably best understood for the dorsal-ventral patterning of the *Drosophila* embryo (reviewed by Stathopoulos and Levine, 2002a).

Dorsal is a member of the Rel family of sequence-specific transcription factors (Steward, 1987). It is initially distributed throughout the cytoplasm of developing oocytes but is transported into nuclei shortly after fertilization (reviewed by Belvin and Anderson, 1996). This regulated nuclear transport process leads to the formation of a broad Dorsal activity gradient, with peak activity in ventral regions and progressively lower levels in lateral and dorsal regions. The Dorsal nuclear gradient is formed by the differential activation of the Toll receptor, which probably depends on an extracellular gradient of the Spätzle ligand (reviewed by Roth, 1994; see Morisato, 2001). The resulting gradient initiates the differentiation of the mesoderm, neurogenic ectoderm, and dorsal ectoderm across the dorsal-ventral axis of the embryo through the differential regulation of ~10–15 previously characterized target genes (reviewed by Rusch and Levine, 1996; see Huang et al., 1997).

Most of the target genes encode sequence-specific transcription factors, including *twi*, *sna*, *vnd*, *brk*, and *zen* (reviewed by Stathopoulos and Levine, 2002a). In order to obtain a more complete understanding of how the Dorsal gradient controls development, Affymetrix chips containing the entire protein coding capacity of the *Drosophila* genome (greater than 13,500 genes) were screened with RNAs extracted from early mutant embryos that contain either no Dorsal protein, uniformly low levels of Dorsal, or uniformly high levels of Dorsal throughout the embryo. Mutant embryos that lack Dorsal overexpress target genes that are normally localized within the dorsal ectoderm, while embryos that contain uniformly low or high levels of Dorsal overexpress neurogenic genes or mesoderm genes, respectively. A total of 353 genes exhibit augmented expression in one or more of these mutant backgrounds; 57 of the genes display significant changes in expression and include as many as 40 new target genes that exhibit localized patterns of expression across the dorsal-ventral axis of wild-type embryos.

Previous studies identified four Dorsal target enhancers; two are activated by peak levels of the Dorsal gradient (*twist* and *snail*), one by intermediate levels (*rhomboid*), and one by low levels (*sog*; see Stathopoulos and Levine, 2002a). New Dorsal target enhancers were characterized in order to investigate the basis for gradient thresholds of gene expression. Three new enhancers were identified, thereby providing at least two enhancers for each of the three major Dorsal gradient thresholds. Bioinformatics methods identified conserved sequence motifs among coordinately regulated enhancers. For example, CACATGT is shared by target enhancers activated by intermediate levels of the Dorsal gradient (*vnd* and *rhomboid*), while GCTGGAA is present in enhancers activated by low levels of the gradient (*Neu4* and *sog*). The new target genes and associated *cis*-regulatory DNAs identified in this study constitute

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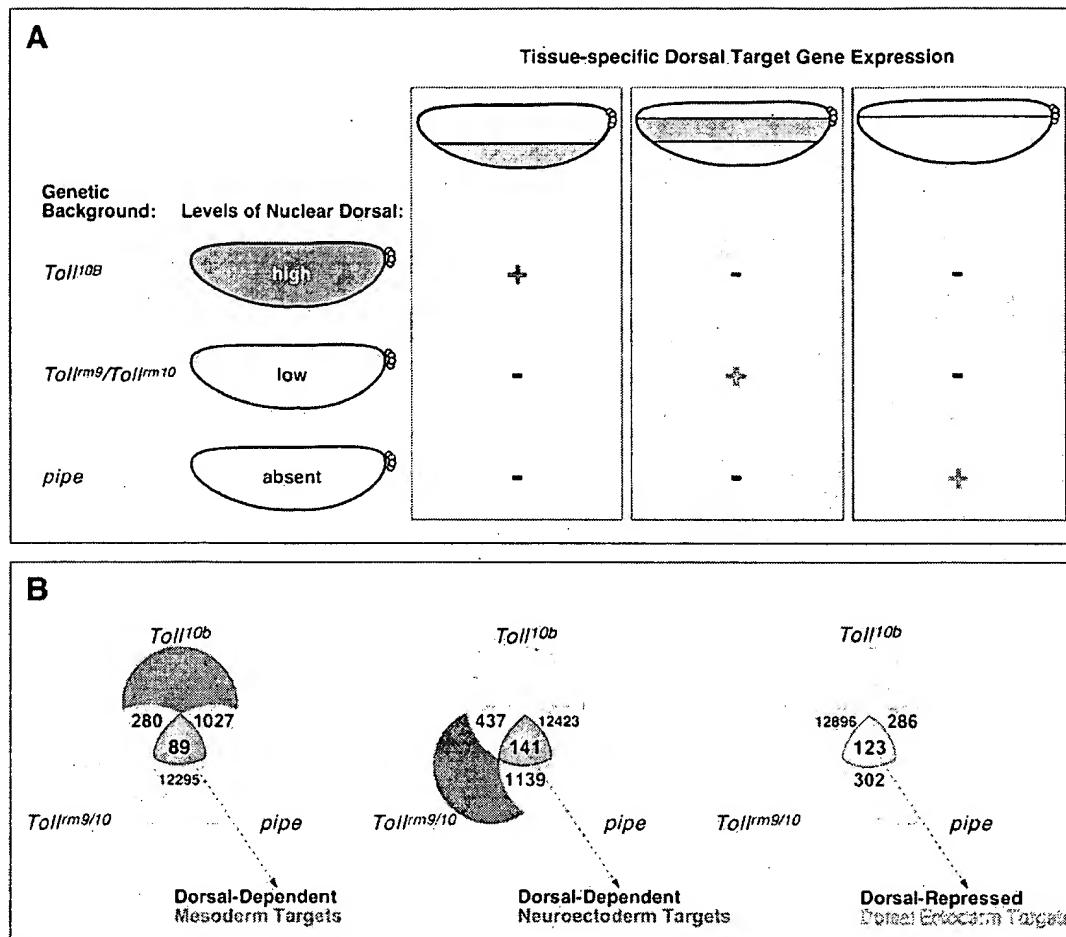


Figure 1. Summary of Microarray Experiments

(A) RNA was isolated from three different genetic backgrounds containing varying amounts of nuclear Dorsal protein. *Toll<sup>10b</sup>* is a dominant mutation in the Toll receptor that leads to high levels of nuclear Dorsal. *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* is a recessive mutation in the Toll receptor that leads to low levels of nuclear Dorsal throughout the early embryo. In *pipe<sup>-</sup>* (*pipe<sup>89</sup>/pipe<sup>89</sup>*) mutant embryos, Dorsal fails to translocate to the nucleus and remains cytoplasmic. Dorsal targets requiring the highest levels of Dorsal for expression are normally only expressed in ventral parts of the embryo, but ubiquitously in the *Toll<sup>10b</sup>* background. Dorsal targets responding to lower levels of nuclear Dorsal are expressed in a broader domain that extends to the lateral regions of the embryo but are absent from the ventral surface due to repression by Snail. These targets are expressed ubiquitously in the *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* background, which contains sufficient levels of Dorsal to promote activation but insufficient levels to activate the repressor Snail. Genes repressed by Dorsal in the dorsal ectoderm are expressed at the dorsal domain of the embryo. These genes are expressed ubiquitously in the *pipe<sup>-</sup>* mutant background due to an absence of nuclear Dorsal. A plus sign indicates the presence of specific targets gene expression in a certain mutant background, whereas a minus sign indicates its absence.

(B) To isolate additional Dorsal target genes, RNA was isolated from each of these three mutants and hybridized to Affymetrix *Drosophila* GeneChips. Genes exhibiting increased expression in response to only the highest levels of nuclear Dorsal (89) were identified by determining which genes are contained within the intersection of three comparisons: those signals that increased at least 3-fold (measured in  $\log_2$ ) in *Toll<sup>10b</sup>* relative to *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* (369) and *pipe* (1116) but changed less than 3-fold in a comparison of *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* versus *pipe* (12384). Genes responding to intermediate levels of nuclear Dorsal (141) were identified by determining which signals increased at least 3-fold in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* relative to *Toll<sup>10b</sup>* (578) and *pipe* (1280) but changed less than 3-fold in *Toll<sup>10b</sup>* versus *pipe* (12564). Genes repressed by Dorsal (123) were identified by determining which signals increased at least 3-fold in *pipe* relative to *Toll<sup>10b</sup>* (409) and *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* (425) but changed less than 3-fold in *Toll<sup>10b</sup>* versus *pipe* (13019). These putative Dorsal targets represent genes expressed in the presumptive mesoderm, neuroectoderm, and dorsal ectoderm, respectively.

one of the most extensive gene regulation networks known for any developmental process.

## Results

Because Dorsal exhibits strict maternal inheritance, it is possible to isolate homogenous populations of mutant

embryos that either lack the gradient (*pipe<sup>-</sup>*) or contain uniformly high (*Toll<sup>10b</sup>*) or low (*Toll<sup>rm9</sup>/Toll<sup>rm10</sup>*) levels of Dorsal in all nuclei (summarized in Figure 1A). Embryos were collected from each of these three classes of mutant females and allowed to develop until cellularization, about 3 hr after fertilization. Dorsal enters nuclei between 90 min to 2 hr after fertilization and activates

target genes such as *twist*, *snail*, and *rhomboid* within the next 30 min to 1 hr (reviewed by Rusch and Levine, 1996). Cellularized embryos should express most of the direct Dorsal target genes, as well as genes that are indirectly regulated by Dorsal (see Discussion). Affymetrix chips containing the complete protein coding capacity of the *Drosophila* genome (greater than 13,500 genes) were separately hybridized with RNAs extracted from each mutant. A total of 353 genes exhibit at least a 3-fold increase in one of the mutant backgrounds (Figure 1B): 89 genes are upregulated in *Toll<sup>10B</sup>* mutants, 141 genes are upregulated in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutants, and 123 genes are upregulated in *pipe<sup>-</sup>/pipe<sup>-</sup>* mutants.

#### Validation of the Microarray Screens

Representative genes that exhibit upregulation in *Toll<sup>10B</sup>*, *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>*, and *pipe<sup>-</sup>/pipe<sup>-</sup>* mutant embryos were selected for further study. These genes fulfill one of the following criteria: availability of an EST, insight into function based on a known protein motif, or conservation in other organisms. Furthermore, the "cutoff" for new putative Dorsal target genes was assessed by examining known genes. For example, most of the known mesoderm-specific genes such as *twist*, *snail*, and *tinman* display at least a ~10-fold increase in *Toll<sup>10B</sup>* mutants as compared with the other genetic backgrounds. Only 19 of the 89 genes that are significantly upregulated (greater than 3-fold) in *Toll<sup>10B</sup>* mutant embryos fulfill this cutoff value (Table 1).

One such gene, *Mes3*, encodes an insulin-like growth factor (*dilp4*) (Brogiolo et al., 2001). In wild-type embryos, the gene is selectively expressed in ventral regions that will form the mesoderm (Figure 2C). The augmented expression of *Mes3* that is seen in *Toll<sup>10B</sup>* mutants (Table 1) can be explained by the marked expansion of the staining pattern in mutant embryos (Figure 2D). A similar expansion is seen for a known gene, *snail*, which is normally expressed in the ventral mesoderm (Figures 2A and 2B).

Dorsal target genes that are expressed in the lateral neurogenic ectoderm, such as *brinker* and *sog* (Table 2), exhibit just 6-fold increases in expression in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutant embryos as compared with the other genetic backgrounds. Only 22 of the 114 genes that are upregulated in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutant embryos fulfilled this cutoff value (Table 2). One of these genes, *Neu3*, encodes a novel member of the ADAM family of endopeptidases (see Qi et al., 1999; Lieber et al., 2002). It is expressed in broad lateral stripes in wild-type embryos (Figure 2G) but is expressed throughout the dorsal-ventral axis of mutant embryos derived from *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* females (Figure 2H). This expanded expression is consistent with the results of the microarray assays, which indicate a 15-fold increase of *Neu3* expression in mutant embryos as compared with wild-type embryos (see Table 2). A similar expansion is observed for a known gene, *sog* (Figures 2E and 2F).

Genes that are specifically expressed within the dorsal ectoderm were identified on the basis of exhibiting augmented expression in *pipe<sup>-</sup>/pipe<sup>-</sup>* embryos (Table 3). *zen* is directly repressed by the Dorsal gradient and is expressed in a progressively more refined pattern during cellularization (Ip et al., 1991). It exhibits a 22-fold

increase in expression in *pipe<sup>-</sup>/pipe<sup>-</sup>* mutant embryos (Table 3). However, other known genes such as *pannier* and *u-shaped* display just 6-fold increases in expression. Only 16 of the 123 genes that are upregulated in *pipe<sup>-</sup>/pipe<sup>-</sup>* mutant embryos fulfill this cutoff value (Table 3). One of the genes, *Ect1*, encodes a TNF (tumor necrosis factor) signaling molecule (reviewed by Baud and Karin, 2001). This gene is normally expressed in the dorsal ectoderm of wild-type embryos (Figure 2K) but is expressed in both dorsal and ventral regions of mutant embryos derived from *pipe<sup>-</sup>/pipe<sup>-</sup>* females (Figure 2L). This expanded staining pattern accounts for the 12-fold increase in the levels of *Ect1* expression observed in the microarray assays (Table 3). A similar expansion is observed for a known gene, *dpp* (Figures 2I and 2J).

#### Localized Expression of New Target Genes

In situ hybridization assays were done for five genes that exhibit at least a 10-fold increase in expression in *Toll<sup>10B</sup>* mutants (Table 1). All five genes display localized expression in the ventral mesoderm in wild-type embryos (Figures 3A–3E). Most of the genes encode proteins that might influence changes in cell size and shape during gastrulation (see Discussion).

Twelve of the genes that exhibit at least a 6-fold increase in expression in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutants were analyzed (Table 2). Seven of these genes exhibit localized expression within the neurogenic ectoderm, whereas five of the genes do not (data not shown). The expression patterns of four of the localized genes are shown (Figures 3F–3I). Two of the genes, *Neu3* and *Neu4*, exhibit more than a 10-fold increase in expression and display broad lateral stripes of expression in the presumptive neurogenic ectoderm (Figures 3H and 3I). In contrast, *Neu1* and *Neu2* exhibit only a 6-fold increase in expression in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutants and display narrower lateral stripes of expression (Figures 3F and 3G). To determine whether genes with smaller increases might respond to different thresholds of the Dorsal gradient, the *Neu5/Sulfated* gene was analyzed since it exhibits just a ~4-fold increase in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* embryos. *Neu5* displays weak, nonuniform staining in the lateral neurogenic ectoderm (Figure 3J).

In situ hybridization assays were done with six different genes that exhibit at least a 6-fold increase in expression in mutant embryos derived from *pipe<sup>-</sup>/pipe<sup>-</sup>* females (see Table 3). Five of the genes that were tested exhibit localized expression within the dorsal ectoderm (Figures 3K–3O), whereas one of the genes does not (data not shown). Among the set of 123 upregulated genes, a homolog of *Ect2*, CG5093, displays 5-fold upregulation. CG5093 exhibits a localized pattern of expression in the dorsal ectoderm that is virtually identical to the *Ect2* pattern (data not shown; Figure 3L).

#### A Tissue-Specific NF- $\kappa$ B Subunit Activates Gene Expression in the Mesoderm

The preceding microarray assays identified a number of genes that are likely to be important for the dorsal-ventral patterning of the early embryo. We selected *Mes4* for further analysis since it encodes a putative mesoderm-specific transcription factor and previous studies suggested that Dorsal is not sufficient for robust



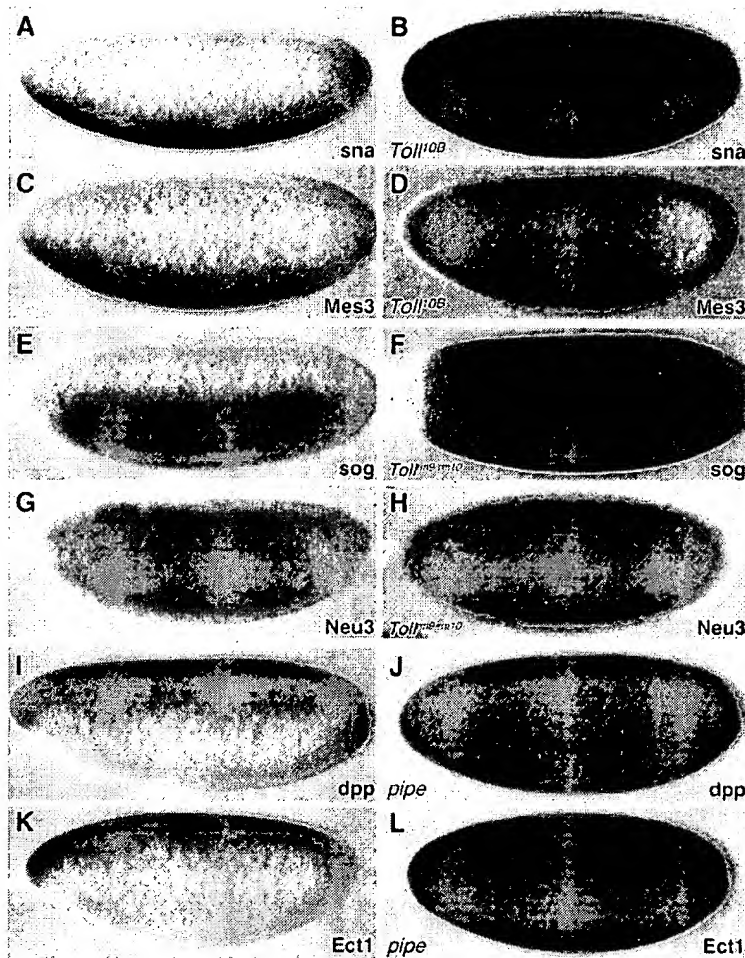
Table 1. Dorsal Targets in the Mesoderm

Mesoderm	Function/Homology	10B/[m9/10]	10B/pipe	pipe/[m9/10]	Confirmed by In Situ	Reference
<b>Known Targets</b>						
fog	unknown	1.1	1.1	-1.0		Costa et al., 1994
heartless (hti)	FGF receptor	14.9	7.7	1.4		Shisido et al., 1993
mei2	MADS-box TF	9.5	7.1	-1.5		Taylor et al., 1995
snail (sna)	transcriptional repressor	3.1	14.2	-5.0		Ip et al., 1992a
tinman (tin)	homeobox TF	10.2	7.0	-2.3		Bodmer et al., 1990
twist (twi)	bHLH TF	63.8	31.3	-1.2		Jiang et al., 1991
zfh1	Zn-finger/homeobox TF	3.6	1.8	2.2		Lai et al., 1991
<b>Microarray-Identified Targets</b>						
Mes1 = RhoL	Rho GTPase	11.9	7.1	-1.7	A, B	Casal and Leptin, 1996
Mes2	ZPW domain	11.0	6.5	-1.7	A	
Mes3 = dilp4	insulin receptor ligand	38.0	38.0	-2.1	A, B	Brogiolo et al., 2001
Mes4	NF- $\kappa$ B homolog	10.7	5.0	1.4	A	
Mes5 = Mdr49	ABC transporter	11.6	24.1	-7.5	A	
Actin57B	cytoskeleton	37.0	17.9	2.2	B	Casal and Leptin, 1996
Argk	arginine kinase	9.8	6.9	-3.5		
Asph	aspartyl $\beta$ -hydroxylase	5.1	11.6	-2.0		
Cyp310a1	cytochrome P450	28.9	7.5	2.1		
glial cells missing	gcm TF	11.7	5.3	1.7		
hoi-polloi	snRNP	9.7	3.7	2.6		
rosy	xanthine dehydrogenase	13.6	6.8	1.3		
stumps	cell signaling	18.9	9.4	1.0	B	Doyle et al., 1989
trachealless	HLH/PAS TF	10.5	4.7	-2.0	B	Casal and Leptin, 1996
	acyl-CoA synthetase	15.0	5.9	2.6		
	Dnase	9.7	5.4	1.0		
	nucleoside hydrolase	8.3	13.5	1.3		
	unknown (Xenopus.hom)	14.8	6.7	5.2		
	unknown (worm.hom)	25.2	5.7	3.2		

TF = transcription factor

A, predicted expression pattern confirmed (this study).

B, predicted expression pattern confirmed (referenced study).



**Figure 2. Expression of Dorsal Target Genes in Wild-Type and Mutant Embryos**

Cellularizing embryos were hybridized with each of the indicated digoxigenin-labeled antisense RNA probes and stained to visualize the gene expression patterns. Embryos are oriented with anterior to the left and dorsal up.

(A and B) *snail (sna)* expression in wild-type (A) and *Toll<sup>108</sup>* embryos (B). *snail* is normally expressed in the ventral mesoderm of wild-type embryos (A) but is ubiquitously expressed in *Toll<sup>108</sup>* mutants (B).

(C and D) *Mes3* expression in wild-type (C) and *Toll<sup>108</sup>* mutant background (D). As seen for *snail*, *Mes3* is normally expressed in the mesoderm, but is greatly expanded in the mutant.

(E and F) *sog* expression in wild-type (E) and *Toll<sup>108</sup>/Toll<sup>109</sup>* (F) embryos. *sog* is normally expressed in lateral stripes in response to even low levels of nuclear Dorsal (E). The gene exhibits ubiquitous expression in mutant embryos (F).

(G and H) *Neu3* expression in wild-type (G) and *Toll<sup>108</sup>/Toll<sup>109</sup>* mutants (H). As seen for *sog*, there is a marked expansion of the expression pattern in mutant embryos.

(I and J) *dpp* expression in wild-type (I) and *pipe<sup>-</sup>* mutant (J) embryos. *Dpp* is normally expressed in the dorsal ectoderm (I) but is derepressed in mutant embryos (J).

(K and L) *Ect1* expression in wild-type (K) and *pipe<sup>-</sup>* (L) mutants. As seen for *dpp*, the expression pattern is expanded in mutant embryos.

activation of gene expression in the mesoderm (Jiang et al., 1991; Ip et al., 1992a; Szymanski and Levine, 1995). *Mes4* encodes a protein that contains a histone-fold multimerization domain and is related to the C subunit of the mammalian NF-Y transcription complex. NF-Y is a trimeric complex that is composed of three subunits, A, B, and C. It binds to conserved CCAAT motifs that are located between -100 bp and -60 bp 5' of the transcription start site in ~30% of all mammalian promoters (Figure 4A). It has been proposed that NF-Y facilitates the binding of TFIID to adjacent core promoter elements such as TATA (reviewed by Mantovani, 1999). There is a curious absence of CCAAT sequences in *Drosophila* promoters, and there have been no reports of an NF-Y complex. However, it is likely that flies contain NF-Y since this complex is conserved in yeast, plants, and vertebrates.

A survey of the *Drosophila* genome reveals clear orthologs of all three NF-Y encoding genes. CG3891 encodes a protein that contains 56/72 AA similarities with the histone-fold domain of the mammalian NF-YA subunit. CG10447 contains 87/118 AA similarities with the histone-fold domain and adjacent regions of the mammalian NF-YB subunit. Finally, CG3075 contains 91/113 similarities with the histone-fold region of the NF-YC

subunit. The *Mes4* gene (CG11301, see Table 1) encodes a divergent copy of the NF-YC subunit that shares 49/78 similarities with the histone-fold region of CG3075. It is considerably more closely related to NF-YC than the other histone-fold proteins in *Drosophila*, such as Dr1, Drap1, TAF<sub>180</sub>, and Chrac-16 (Aoyagi and Wassarman, 2000).

The three primary NF-Y genes are maternally expressed, suggesting their encoded RNAs are ubiquitously distributed throughout early embryos (BDGP; <http://www.fruitfly.org/cgi-bin/ex/insitu.pl>). However, expression of the C subunit gene is rapidly lost in early embryos, at the time when the *Mes4* gene is first activated in the presumptive mesoderm (Figure 4B). This raises the possibility that a functional NF-Y complex is made only in the mesoderm during gastrulation and germband elongation. The *Mes4* staining pattern is initially uniform but exhibits segmental modulations along the anterior-posterior axis by the completion of cellularization. Expression is restricted to the ventral-most 18–20 cells, which coincides with the presumptive mesoderm (Figure 4H). Staining persists in the mesoderm during invagination and germband elongation (Figures 4C and 4D). At the time of invagination, *Mes4* transcripts exhibit clear segmental repeats and the staining

Table 2. Dorsal Targets in the Neuroectoderm

Neuroectoderm	Function/Homology	[rm9/10]/10B	pipe/10B	[rm9/10]/pipe	Confirmed by In Situ	Reference
<b>Known Targets</b>						
brinker (brk)	transcriptional repressor	4.9	-1.2	5.7		Jazwinska et al., 1999
ind	homeobox TF	17.8	-3.9	17.6		Weiss et al., 1998
lethal of scute (T3)	HLH TF	4.2	1.4	3.0		Romani et al., 1987
rhomboid (rho)	serine-type peptidase	2.9	-1.2	1.9		Ip et al., 1992b
single-minded (sim)	HLH/PAS TF	-4.2	-2.2	1.5		Kasai et al., 1998
sog	growth factor	6.4	-1.5	5.1		Francois et al., 1994
vnd	homeobox TF	4.0	-2.2	3.1		McDonald et al., 1998
<b>Microarray-Identified Targets</b>						
Neu1 = Dscam	axon guidance receptor	5.5	1.6	2.0	A	
Neu2	zinc-finger protein	5.1	-1.1	5.7	A	
Neu3	ADAM metalloprotease	15.4	-1.1	8.9	A	
Neu4	unknown function	11.1	-1.1	6.3	A	
Neu5 = sulfated	sulfotransferase	2.2	-2.3	3.7	A	below cutoff
echinoid	cell adhesion	7.0	-2.7	5.3	NL	
leak	axon guidance receptor	6.9	-2.8	7.7	NL	
patched	receptor	11.9	-2.9	8.1	NL	
PGRP-SC2	defense/immunity	6.2	-2.0	5.8		
Scabrous	receptor ligand	19.7	3.1	6.9	A, B	Mlodzik et al., 1990
Socs36E	cell signaling	13.5	-2.1	6.7		
SoxNeuro	HMG-box TF	18.0	-3.1	11.2	A, B	
warts	kinase	4.4	-2.7	6.0	NL	Cremazy et al., 2000
	calcium binding	5.6	-1.8	6.5		
	methyltransferase	12.0	-2.1	7.4		
	serine protease inhibitor	8.2	-1.8	5.3	A	
	myosin binding	6.8	-2.4	5.2		
	WD40 repeats	7.8	-1.9	5.0	NL	
	myosin/kinesin motor	7.8	-1.8	5.9	NL	
	unknown (embryoEST)	8.2	-2.3	6.7		
	unknown (homologs)	7.1	-2.4	5.2		
	G-protein signaling	7.6	-1.9	5.1		
	zinc-finger protein	6.6	-2.6	5.3		

TF = transcription factor

A, predicted expression pattern confirmed (this study).

B, predicted expression pattern confirmed (referenced study).

NL = not localized

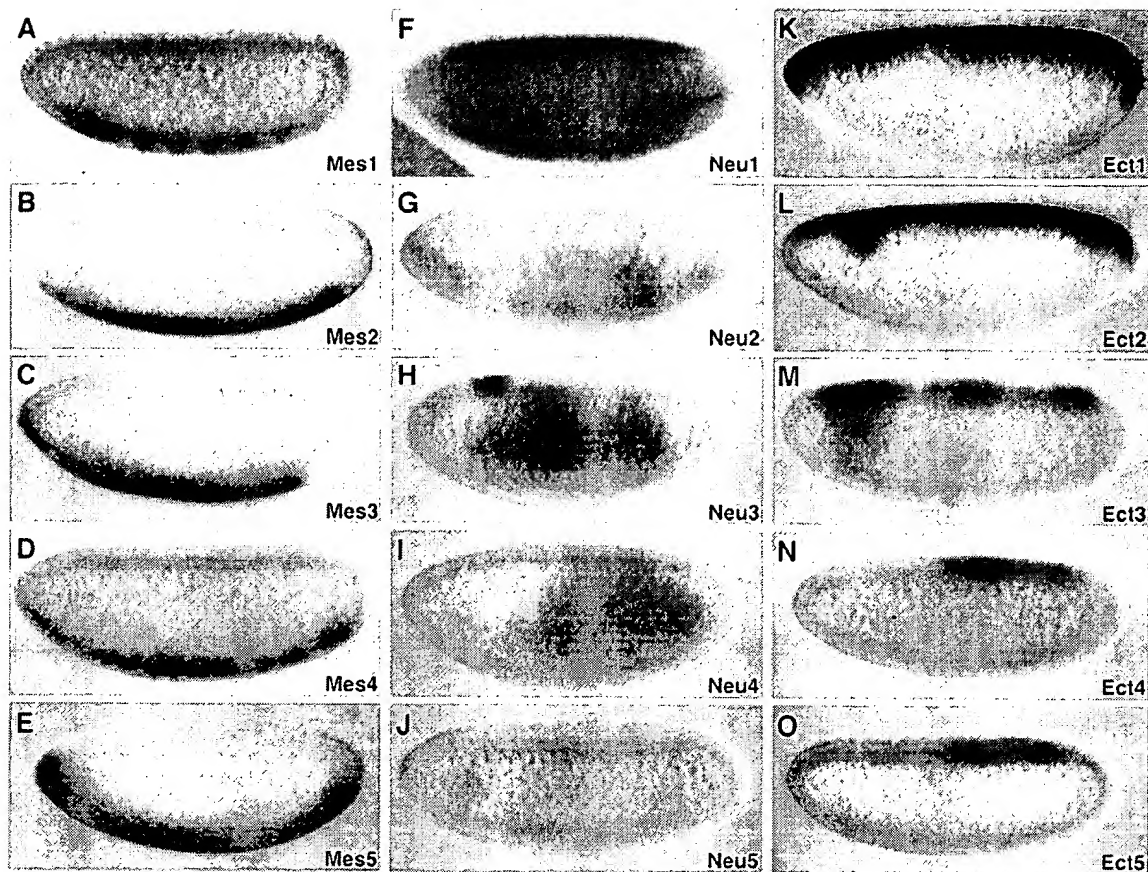
Table 3. Dorsal Targets in the Dorsal Ectoderm

Dorsal Ectoderm	Function/Homology	[m9/10/10B]	pipe/10B	pipe/[m9/10]	Confirmed by In Situ	Reference
<b>Known Targets</b>						
decapentaplegic (dpp)	TGF- $\beta$ receptor ligand	2.4	5.1	3.6		St. Johnston and Gelbart, 1987
pannier (pnr)	GATA TF	1.4	6.4	6.3		Winick et al., 1993
race	peptidyl dipeptidase A	-1.7	3.3	5.4		Tatei et al., 1995
tolloid (tld)	endopeptidase	7.1	11.4	4.4		Kirov et al., 1994
tailup (tup)	homeobox TF	1.3	3.2	3.2		Frank and Rushlow, 1996
u-shaped (ush)	Zn-finger TF	1.2	6.0	5.2		Frank and Rushlow, 1996
zerknüllt (zen)	homeobox TF	-1.0	21.9	22.7		Doyle et al., 1989
<b>Microarray-Identified Targets</b>						
Ect1	TNF receptor ligand	2.1	11.9	11.4	A	
Ect2 = Dorsocross	T domain TF	1.1	28.6	32.5	A	
Ect3	galactosidase	-1.4	15.5	20.9	A	
Ect4	SAM, TIR, Pro-rich domains	-1.0	6.4	6.6	A	
Ect5 = C15	homeobox TF	1.6	6.8	5.2	A	
Adult cuticle protein1	structural cuticle protein	2.2	7.3	6.0		
Lcp65Ag2	structural cuticle protein	-3.1	13.3	19.9		
	T domain TF	-1.0	4.9	5.1	A	below cutoff
	unknown (embryo EST)	1.6	17.2	15.5		
	unknown (embryo EST)	1.6	11.4	11.2		
	phospholipid binding	-1.4	6.1	8.6	NL	
	ligand binding/carrier	1.9	6.0	6.4		
	alpha-mannosidase	2.6	6.4	5.4		
	bipartite nls	-1.6	6.1	6.4		
	unknown (embryo EST)	1.2	10.2	6.8		
	bipartite nls; Pro-rich	1.6	6.5	4.0		
	unknown (embryo EST)	1.8	7.6	7.2		

TF = transcription factor

A, predicted expression pattern confirmed (this study).

NL = not localized



**Figure 3. Expression of New Dorsal Target Genes**

Cellularizing embryos were hybridized with each of the indicated digoxigenin-labeled antisense RNA probes and are oriented with anterior to the left and dorsal up.

(A–E) Genes expressed in the presumptive mesoderm responding to only the highest levels of nuclear Dorsal. (A) *Mes1/RhoL*, (B) *Mes2/CG11100*, (C) *Mes3/dilp4*, (D) *Mes4/CG11301*, and (E) *Mes5/Mdr49*.

(F–J) Genes expressed in the presumptive neuroectoderm responding to low levels of nuclear Dorsal. (F) *Neu1/Dscam*, (G) *Neu2/CG7204*, (H) *Neu3/CG7649*, (I) *Neu4/CG12443*, and (J) *Neu5/sulfated*.

(K–O) Genes expressed in the presumptive dorsal ectoderm presumably repressed by nuclear Dorsal. (K) *Ect1/CG12919*, (L) *Ect2/Dorsocross*, (M) *Ect3/CG3132*, (N) *Ect4/CG7915*, and (O) *Ect5/C15*.

pattern consists of six “stripes” between the cephalic furrow and presumptive abdomen; a seventh stripe is seen just anterior to the furrow (Figure 4H).

The distinctive *Mes4* staining pattern provides evidence that it functions as an activator of *snail* (see below). This was directly tested by placing the *Mes4* protein coding region under the control of the *Krüppel* 5' regulatory region. Transgenic embryos that contain the *Krüppel-Mes4* fusion gene exhibit an ectopic band of staining in central regions, in addition to the normal pattern in ventral regions (Figure 4E; compare with Figure 4B). The ectopic *Mes4* expression pattern leads to the misexpression of *snail*. Normally, *snail* expression is restricted to ventral regions that form the mesoderm (Figure 4F), but it is weakly misexpressed in central regions of transgenic embryos carrying the *Krüppel-Mes4* fusion gene (Figure 4G).

*snail* is normally expressed uniformly, but exhibits nonuniform stripes in *twist<sup>-</sup>/twist<sup>-</sup>* mutant embryos (Fig-

ure 4I; Ip et al., 1992a). These stripes are very similar to those seen for the normal *Mes4* expression pattern (compare Figures 4H and 4I). Thus, in the absence of Twist, Dorsal and *Mes4* might function as the primary activators of the *snail* expression pattern.

#### Identification of New Dorsal Target Enhancers

Previous studies identified only four Dorsal target enhancers (reviewed in Stathopoulos and Levine, 2002a; see Markstein et al., 2002). This is not a sufficient collection to determine whether enhancers that respond to similar thresholds of the Dorsal gradient contain shared *cis*-regulatory elements. New enhancers were identified for the *Mes3* and *Neu4* genes by scanning ~25 kb of associated genomic DNA for clusters of Dorsal recognition sequences. Unlike previous whole-genome screens for clusters of optimal Dorsal binding sites (Markstein et al., 2002), the search for putative enhancers associated with known genes is much less stringent and permits

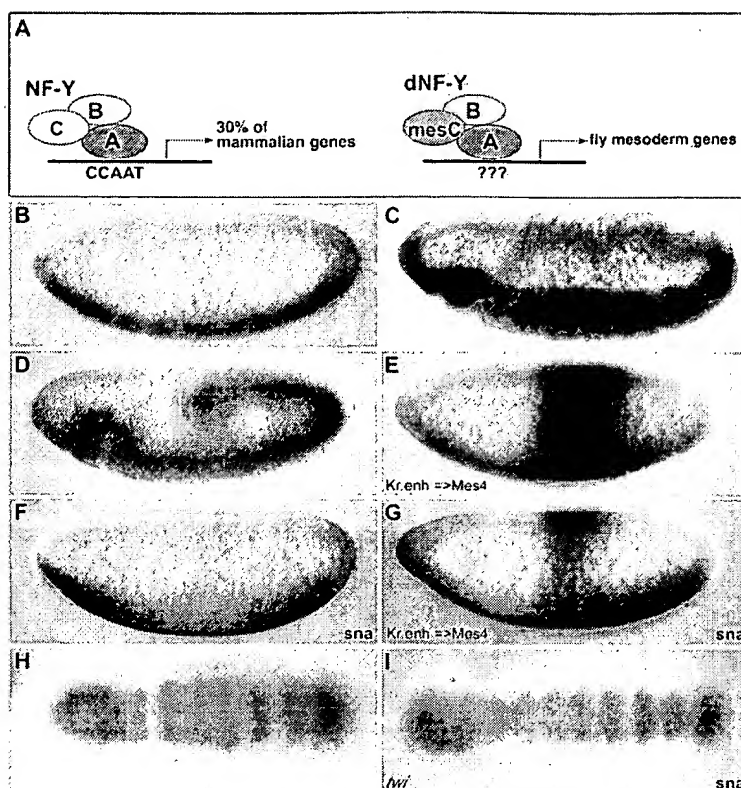


Figure 4. *Mes4*, a Novel Dorsal Target Gene Identified by Microarray, Contains Homology to NF-YC and Is Involved in Regulating *snail* Expression

(A) NF-Y is a heterotrimeric protein composed of subunits A, B, and C that binds to CCAAT motifs present from -100 to -60 bp upstream of the major transcriptional start site in 30% of mammalian promoters. *Drosophila* contains orthologs of all three subunits, and *Mes4* is the first described tissue-specific metazoan NF-YC homolog. We suggest that a tissue-specific NF-Y complex containing the *Mes4* subunit helps activate the expression of mesoderm-specific genes, such as *snail*. (B-I) In situ hybridization experiments were performed using antisense probes to either *Mes4* (B-E and H) or *snail* (*sna*; F, G, and I). *Mes4* is normally expressed in the presumptive mesoderm, in ventral regions of the embryo. (B) and (C) are lateral views of *Mes4* expression in mid-nuclear cleavage cycle 14 (B) and gastrulating (C) embryos. The expression pattern exhibits pair-rule modulations along the anterior-posterior axis (H; ventral view) and persists in the mesoderm through germband elongation (D). To study its function, *Mes4* was cloned into a P element insertion vector that promotes ectopic expression in the region of *Krüppel* expression (*Kr-FSF*; see Experimental Procedures) and was used to generate transgenic lines that misexpress this gene (*Kr.enh* => *Mes4*; compare [E] with [B]). *snail*, which is normally expressed only

in ventral regions of the embryo in the presumptive mesoderm (F), is ectopically expressed in the *Krüppel* domain when *Mes4* is misexpressed (compare [G] with [F]). Interestingly, the fact that *snail* expression, which normally extends 18-20 cells in a broad ventral band in wild-type embryos, is reduced in width and exhibits stripes of expression in a *twist* (*twi*) mutant background (I) implies that *Mes4* may regulate the expression of *snail* in the absence of *Twist*.

the use of degenerate, low-affinity Dorsal binding sites.

We investigated the feasibility of this approach by analyzing the previously identified *ventral nervous system defective* (*vnd*) gene. Recent studies identified multiple enhancers in the 5' flanking region that mediate *vnd* expression in specific neuroblasts of advanced-stage embryos (Shao et al., 2002). However, these enhancers do not direct lateral stripes of *vnd* expression within the ventral neurogenic ectoderm. The best cluster of potential Dorsal binding sites in the *vnd* genomic interval is located within the first intron (Figure 5A). A ~1.7 kb DNA fragment that encompasses these binding sites was placed 5' of a minimal *eve-lacZ* fusion gene and expressed in transgenic embryos. The fusion gene exhibits lateral stripes of *lacZ* expression that are virtually identical to the endogenous *vnd* expression pattern (Figures 5B and 5C).

A cluster of four low-affinity Dorsal binding sites were identified in a 260 bp region of the *Mes3* (*dilp4*) 5' flanking sequence (Figure 5D). The *Mes3* gene is selectively expressed in the ventral mesoderm (Figures 2C and 3C), and the associated putative Dorsal binding sites are similar to those seen in the 5' flanking regions of the *snail* and *twist* genes (Jiang et al., 1991; Ip et al., 1992a). The 260 bp fragment from *Mes3* was placed 5' of a minimal *eve-lacZ* fusion gene and expressed in transgenic embryos. *LacZ* staining is detected in ventral regions at the onset of nuclear cleavage cycle 14 (Figure 5E). This staining pattern persists during cellularization,

gastrulation, and germband elongation (Figure 5F). The expression profile generated by the 260 bp DNA fragment is similar to that observed for the endogenous *Mes3* gene (e.g., Figure 3C). For example, *lacZ* staining is excluded from the posterior pole as seen for the endogenous pattern (compare Figure 5E with Figure 3C). Thus, it was possible to identify an authentic mesoderm-specific enhancer from the *Mes3* 5' flanking region by simply identifying the best potential Dorsal binding cluster in the vicinity of the gene.

A new neurogenic enhancer was identified in the 5' flanking region of the *Neu4* gene, which is expressed in broad lateral stripes that encompass both ventral and dorsal regions of the neurogenic ectoderm in the presumptive thorax and abdomen (Figure 3I). Previous studies identified 16 regions in the entire genome that contain 3 or 4 optimal Dorsal binding sites within a stretch of 400 bp or less (Markstein et al., 2002). The highest density binding cluster is located 15.2 kb 5' of the *Neu4* gene. There are three evenly spaced binding sites within a stretch of just 75 bp (Figure 5G). A 500 bp fragment that encompasses these sites was placed 5' of an *eve-lacZ* fusion gene and expressed in transgenic embryos. *LacZ* staining is detected in the neurogenic ectoderm at the onset of nuclear cleavage cycle 14 (Figure 5H). As seen for the endogenous gene, staining is strongest in the presumptive thorax and abdomen (see Figure 3I). This staining pattern persists during gastrulation and germband elongation (Figure 5I). It is possible

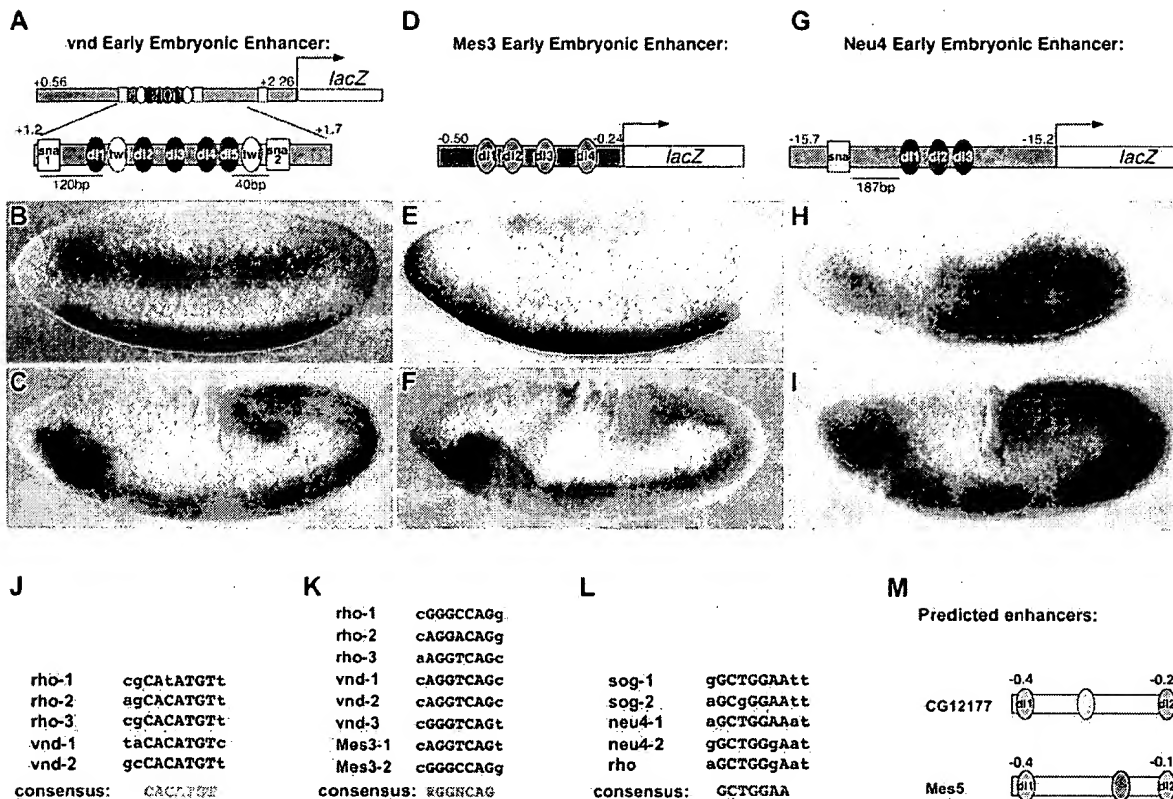


Figure 5. The Identification of New Dorsal Target Enhancers and Novel *cis*-Regulatory Elements

(A, D, and G) Putative enhancers were initially identified by the presence of potential clusters of Dorsal binding sites.

(A–C) The best cluster in the *vnd* genomic region is located in the first intron, between 560 bp and 2.26 kb downstream of the transcription start site (A). The diagram shows the location of putative high-affinity Dorsal binding sites (black ovals), two Twist sites (white ovals), as well as two potential Snail repressor sites (white squares) that might keep the gene off in the ventral mesoderm. This 1.7 kb DNA fragment was placed 5' of an *eve-lacZ* fusion gene and expressed in transgenic embryos. Embryos were hybridized with a digoxigenin-labeled *lacZ* RNA probe. Staining is initially detected in lateral stripes that straddle the mesoderm (B). These stripes correspond to the ventral half of the neurogenic ectoderm and persist during gastrulation (data not shown) and germband elongation (C).

(D–F) The best Dorsal binding cluster in the *Mes3/dilp4* region is located between 500 bp and 240 bp 5' of the transcription start site. The diagram shows the location of low-affinity Dorsal binding sites (gray ovals). This 260 bp fragment was placed 5' of the *eve-lacZ* fusion gene and expressed in transgenic embryos (D). Staining is detected in the presumptive mesoderm during mid-nuclear cleavage cycle 14 (data not shown) and persists during cellularization (E) and gastrulation (F). Staining is restricted to the ventral mesoderm.

(G–I) The best Dorsal binding cluster associated with the *Neu4* gene was identified in a whole-genome search for optimal Dorsal clusters. The highest density cluster (three optimal Dorsal sites in 75 bp; black ovals) is located ~15 kb 5' of the *Neu4* transcription start site (G). A 500 bp fragment that encompasses these sites was placed 5' of the *eve-lacZ* reporter gene and expressed in transgenic embryos. Staining is detected in broad lateral stripes in cellularizing embryos (H) and persists during gastrulation and germband elongation (I). Staining is excluded from the ventral mesoderm, possibly by binding of the Snail repressor to a site located in the 500 bp *Neu4* 5' fragment (see diagram in [G]; white square).

(J–M) Comparative analyses of two enhancers from each of these representative thresholds led to the identification of conserved sequence elements. Exact matches to the computed consensus are depicted in uppercase; sequences not conforming to the consensus in lowercase.

(J) CACATGT is present in *rho* and *vnd* but not the other enhancers.

(K) RGGNCAG is present in multiple enhancers (*rho*, *vnd*, and *Mes3*).

(L) Perfect matches to GCTGGAA are present in *sog* and *Neu4*; a divergent copy of this sequence is also present in *rho*.

(M) Using these novel *cis*-regulatory motifs, enhancers were predicted in other putative Dorsal targets that contain only two low-affinity Dorsal sites. These include CG12177, which contains low-affinity Dorsal binding sites and one CACATGT site (blue oval), and *Mes5*, which contains low-affinity Dorsal binding sites and one RGGNCAG site (red oval).

that *Neu4* is repressed in the ventral mesoderm by the Snail repressor since the 500 bp enhancer contains at least one optimal Snail binding site (Figure 5G).

#### Identification of Conserved Sequence Motifs

Bioinformatics methods were used to identify shared sequence motifs among the expanded collection of Dor-

sal target enhancers (see Experimental Procedures). Particular efforts focused on coordinately regulated enhancers that respond to similar levels of the Dorsal gradient. The newly identified *vnd* enhancer directs an expression pattern that is virtually identical to the one produced by the 300 bp *rhomboid* NEE (Ip et al., 1992b). Both enhancers are activated by intermediate levels of



the Dorsal gradient and direct lateral stripes of expression within ventral regions of the neurogenic ectoderm. In addition to Dorsal binding sites, the two enhancers share an additional sequence motif: CACATGT. There are multiple copies of this motif in each enhancer (Figure 5J). The motif is probably recognized by the bHLH protein Twist, which is distributed in a steep gradient in ventral regions of the neurogenic ectoderm (Kosman et al., 1991). Clustered Dorsal binding sites and the CACATGT motif are observed within the intron of the *Dscam* (*Neu1*) gene, which also exhibits lateral stripes in the ventral neurogenic ectoderm (see Figure 3F).

The newly identified *Neu4* enhancer directs broad lateral stripes of gene expression in response to low levels of the Dorsal gradient. The staining pattern is very similar to the one produced by the 400 bp *sog* intronic enhancer (see Markstein et al., 2002). Both the *Neu4* and *sog* enhancers contain a series of evenly spaced, optimal Dorsal binding sites (GGG-W<sub>4-5</sub>-CCC, where W = A or T). They also share a novel sequence motif, GCTGGAA (Figure 5L). There are two copies of this motif in each enhancer, but it is generally absent in Dorsal target enhancers that are activated by higher concentrations of the Dorsal gradient (although there is one copy of the motif in the *rhomboid* NEE). It is conceivable that the GCTGGAA motif interacts with an unknown transcription factor, which permits low levels of the Dorsal gradient to activate gene expression in dorsal regions of the neurogenic ectoderm.

A third sequence motif was identified in the *vnd* enhancer, the *rhomboid* NEE, and the *Mes3* enhancer: RGGNCAG (Figure 5K). These enhancers are activated by either high or intermediate levels of the Dorsal gradient. This new motif helps identify putative mesoderm enhancers, which are often activated by degenerate, low-affinity Dorsal binding sites that are difficult to discern. For example, a 300 bp DNA fragment located 110 bp 5' of the *Mes5* transcription start site contains two putative Dorsal binding sites and one copy of the RGGNCAG motif (Figure 5M). Similarly, a putative enhancer was identified in the 5' regulatory region of *CG12177* (see Table 1) based on the occurrence of low-affinity Dorsal binding sites and a copy of the CACATGT motif (Figure 5M). It is conceivable that *CG12177* is expressed in both the ventral mesoderm and ventral regions of the neurogenic ectoderm (data not shown).

## Discussion

Microarray assays identified a large number of new Dorsal target genes. Approximately two-thirds of the genes that were tested (19/25) exhibit localized patterns of gene expression across the dorsal-ventral axis of wild-type embryos. Moreover, an additional 32 untested genes fulfill the cutoff criteria (Tables 1–3), and ~20 (two-thirds) would be expected to display localized expression. Thus, this study identified as many as 40 new Dorsal target genes. It is likely that at least half correspond to direct targets of the Dorsal gradient since they are activated during early embryogenesis, within an hour of Dorsal nuclear transport.

There have been earlier attempts to identify genes

that are specifically expressed in the mesoderm (Casal and Leptin, 1996; Furlong et al., 2001). Several such genes were identified, including *Mes1*. However, the screens were not performed to saturation and many of the genes are activated in mesoderm derivatives after the Dorsal gradient initiates dorsal-ventral patterning. In contrast, the present study focused on early embryos in order to identify direct target genes. In addition, *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* mutants were used to isolate genes that are selectively expressed in the neurogenic ectoderm, while the *pipe* mutant permitted the identification of genes that are selectively expressed in the dorsal ectoderm.

Mesoderm cells undergo extensive changes in cell shape during gastrulation (reviewed by Leptin and Roth, 1994). Previous studies identified a putative G-coupled signaling pathway that influences changes in cell shape, including *folded gastrulation*, a Rho GTPase (DRho-GEF2), and *concertina*, an  $\alpha$  subunit of a heteromeric G protein complex (reviewed by Leptin and Roth, 1994; see Morize et al., 1998; Hacker and Perrimon, 1998). Many of the mesoderm genes that were identified in this screen (Table 1) encode proteins implicated in changes in cell growth and proliferation, including a Rho GTPase (*Mes1*), an insulin-like growth factor (*Mes3*), an ABC transporter (*Mes5*), acyl-CoA synthetase (CG4500), and a nucleoside hydrolase (CG12177). It seems likely that one or more of these genes influence changes in cell shape or size, possibly by interacting with the *fog* signaling pathway.

Evidence that at least one of the new mesoderm target genes has a function in the early embryo was obtained for *Mes4*. It is the first example of a tissue-specific NF-Y subunit in metazoans. It joins a growing list of general transcription factors that have duplicated to produce a tissue-specific variant, which controls the differentiation of specific cell types. Other examples include tissue-specific TAFs, such as *cannonball*, which is a TAF<sub>80</sub> derivative that is required for spermatogenesis (Hiller et al., 2001), and TAF<sub>1105</sub>, a TAF<sub>130</sub> derivative that is required for the differentiation of follicle cells in the mouse ovary (Freiman et al., 2001). TAF<sub>80</sub> and *Mes4* contain histone-fold dimerization motifs, which are conserved in a small subset of general transcription factors that function at or near the core promoter (e.g., Aoyagi and Wassarman, 2000).

*Ect1* represents the best-conserved TNF homolog in the fly genome. TNFs have been implicated as key mediators of JNK signaling and apoptosis in a variety of mammalian tissues (reviewed by Baud and Karin, 2001). *Ect1* might play a similar role in *Drosophila* since the gene is specifically expressed in the presumptive amnioserosa during gastrulation. The *Drosophila* homologs of *fos* and *jun* exhibit similar patterns of expression (Rusch and Levine, 1997; Riesgo-Escovar and Hafen, 1997), thereby raising the possibility that TNF triggers the histolysis of the amnioserosa by inducing JNK signaling (Baud and Karin, 2001). *Ect1* corresponds to a recently identified gene called *Eiger*, which was shown to trigger cell death upon overexpression in the adult eye (Igaki et al., 2002).

The relatively late onset of *Ect2* expression (see Figure 3L) suggests that it might be regulated by the Dpp activ-

ity gradient present in the dorsal ectoderm of cellularized embryos (reviewed by Podos and Ferguson, 1999). *Ect2* encodes a T domain transcription factor, thereby raising the possibility that there is an evolutionarily conserved link between TGF- $\beta$  signaling and T box transcription factors. TGF- $\beta$  gradients regulate T box genes in the *Xenopus* animal cap (reviewed by Gurdon and Bourillot, 2001) and the *Drosophila* wing imaginal disk (reviewed by Strigini and Cohen, 1999). A second T box gene, CG5093, exhibits an expression pattern that is virtually identical to the *Ect2* pattern (see Table 3). Potential redundancy in the activities of the *Ect2* and CG5093 genes might explain why these genes were not identified in previous genetic screens. Similar arguments apply to some of the other genes identified in this screen, such as *Mes1* and *Mes4*. Perhaps mutations in the *Mes1* Rho GTPase are compensated by the maternal expression of the *DRhoGEF2* GTPase (Hacker and Perrimon, 1998), while mutations in *Mes4* are compensated by the general NF-YC subunit encoded by CG3075.

The combination of microarray assays and bioinformatics methods provided a highly effective means for identifying new target enhancers and potential *cis*-regulatory elements that respond to different thresholds of the Dorsal gradient. The analysis of coordinately regulated enhancers provided an opportunity to identify shared sequence elements that might help specify different threshold readouts of the Dorsal gradient. The identification of the CACATGT motif in target enhancers that are activated by intermediate levels of the gradient reinforce the view that Twist is not a dedicated mesoderm determinant, but is also essential for specifying the ventral neurogenic ectoderm (see Stathopoulos and Levine, 2002b). The RGGNCAG motif facilitates the identification of mesoderm enhancers, which are generally regulated by poorly conserved, low-affinity Dorsal binding sites. Future studies will determine whether the newly identified GCTGGAA motif is essential for the activation of target enhancers by the lowest levels of the Dorsal gradient.

In terms of sheer number of potential target genes and associated *cis*-regulatory DNAs, Dorsal represents the most thoroughly characterized morphogen in development. Target genes were identified for every dorsal-ventral patterning threshold by analyzing mutant embryos that express different concentrations of the Dorsal protein. In principle, a similar strategy could be used for other patterning processes, such as the specification of different neurons in the vertebrate neural tube by a gradient of Sonic Hedgehog (Jessell, 2000).

#### Experimental Procedures

##### *Drosophila* Stocks and Genetic Crosses

Flies containing a dominant gain-of-function mutation of the maternal gene *Toll*, *Toll<sup>100</sup>*, were obtained from S. Govind (Schneider et al., 1991). Flies containing recessive *Toll* mutations, *Toll<sup>rm9</sup>* and *Toll<sup>rm10</sup>*, were obtained from K. Anderson (Schneider et al., 1991). Flies containing recessive *pipe* mutations, *pipe<sup>386</sup>* and *pipe<sup>664</sup>*, were obtained from D. Stein (Sen et al., 1998). A transgenic line homozygous for a P[ry<sup>+</sup>  $\beta$ 2-tubulin-*flp*] insertion was provided by G. Struhl. *twist<sup>-</sup>* flies (*cn twi bw sp/CyO*; #2381) were obtained from the Bloomington Stock Center. Other transgenic lines described in this work were generated using *yw<sup>67c23</sup>* flies.

Females of the genotype *Toll<sup>100</sup>/+* were obtained directly from the balanced stock (*Toll<sup>100</sup>/TM3 Sb Ser* and *Toll<sup>100</sup>/OR60*). To generate *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* females, females of the genotype *Toll<sup>rm9</sup>/TM3 Sb* were mated with males of the genotype *Toll<sup>rm9</sup>/TM3 Ser*. Non-Sb, non-Ser females of the genotype *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* were selected. To generate *pipe<sup>-</sup>/pipe<sup>-</sup>* females, *pipe<sup>664</sup>/TM3 Sb* females were mated with males of the genotype *pipe<sup>386</sup>/TM3 Sb*. Non-Sb females of the genotype *pipe<sup>386</sup>/pipe<sup>664</sup>* were selected. Embryos were collected from females of the selected genotypes, either *Toll<sup>100</sup>/+*, *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>*, or *pipe<sup>386</sup>/pipe<sup>664</sup>*.

##### Transgenic Lines

To ectopically express *Mes4* within the *Krüppel* domain, females containing the misexpression construct *Kr-FSF-Mes4* were mated with males carrying the  $\beta$ 2-tubulin-*flp* gene to obtain males containing both transgenes. In these males,  $\beta$ 2-tubulin-*flp* catalyzes the activation of the misexpression construct by the spermatocyte-specific removal of stop codons contained within a *flp*-out cassette (FSF). These males were mated to *yw<sup>67c23</sup>* females to establish a flipped line, which we determined was viable. Embryos were collected from these homozygous, flipped lines and analyzed by in situ hybridization (Tautz and Pfeifle, 1989; Jiang et al., 1991). To analyze the *vnd*, *Mes3*, and *Neu4* enhancer reporters, embryos were collected from transformants and analyzed by in situ hybridization using a *lacZ* antisense RNA probe. For the *vnd* enhancer transgenic lines, one transformant was analyzed. For *Kr-FSF-Mes4*, *Mes3*, *Neu4* transgenic lines, at least three transformants were analyzed for each. The staining patterns depicted in figures represents the staining pattern observed for the majority of embryos examined for each line.

#### Microarray Experiments

##### Sample Preparation

Two hour embryo collections were made from either *Toll<sup>100</sup>*, *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>*, or *pipe<sup>386</sup>/pipe<sup>664</sup>* mutant females on apple juice/yeast plates at 25°C. Plates were removed and the embryos were aged an additional 2 hr at 25°C. Carefully staged embryos which had been aged 2–4 hr in this manner were collected, dechorionated, and frozen in liquid nitrogen for storage at –80°C until RNA was to be isolated. Multiple collections from different days were pooled for each sample in order to better normalize the age of these embryo populations. Once sufficient amounts of embryos had been collected (~500 embryos), total RNA was extracted from them using Trizol Reagent (GIBCO-BRL) according to the manufacturer's protocol. For each sample, 100  $\mu$ g of total RNA was further purified using the RNeasy Mini Kit (Qiagen) following the RNeasy Mini Protocol for RNA cleanup. Total RNA was prepared independently three times from embryos of each genetic background.

##### Probe Preparation

cDNA synthesis was carried out as described in the Expression Analysis Technical Manual (Affymetrix) using 7  $\mu$ g of total RNA for each sample. The cRNA reactions were carried out using the BioArray High-Yield Transcript Labeling kit (Enzo). 20  $\mu$ g of labeled cRNA was fragmented for 35 min at 94°C using fragmentation buffer (200 mM Tris-acetate [pH 8.1], 500 mM KOAc, 150 mM MgOAc). Affymetrix high-density oligonucleotide arrays for *Drosophila melanogaster* were probed, hybridized, stained, and washed according to the manufacturer's protocol. Greater than 13,500 gene sequences predicted from the annotation of the *Drosophila* genome (version 1) are represented on the array.

##### Data Analysis

Hybridized arrays were scanned using Affymetrix Microarray Suite software as described in the manufacturer's protocol. GeneChip Analysis Suite Software was used to normalize the data contained in each experimental GeneChip.dat file, creating GeneChip.chp files for each experiment. For comparison analysis, GeneChip.dat files were analyzed relative to the GeneChip.chp file defined as baseline. In this way, pair-wise comparisons were made using the GeneChip program between the *Toll<sup>100</sup>* and *pipe<sup>-</sup>/pipe<sup>-</sup>*, the *Toll<sup>100</sup>* and *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>*, and the *pipe<sup>-</sup>/pipe<sup>-</sup>* and *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* microarray data. Data output of these comparisons was formatted in Microsoft Excel and then imported into FileMaker Pro for further analysis. Fold-differences are reported in log<sub>2</sub> such that increasing and decreasing levels of RNA can be compared directly. As discussed in Casal and Leptin

(1996), we examined the levels of a gene uniformly expressed in the embryo  $\beta$ Tub56D (Natzle and McCarthy, 1984) as a control for our RNA samples. We found that the levels of this transcript changed less than 1-fold for each microarray experimental comparison, as expected for a uniformly expressed gene.

#### Plasmid Construction, P Element-Mediated Germline Transformation, and Whole-Mount In Situ Hybridization

An ~1.7 kb genomic DNA fragment located 560 bp downstream of the *vnd* start codon was amplified from *Drosophila melanogaster* genomic DNA using polymerase chain reaction (PCR) and the primers 5'-gggtaagcacaaggattccaatg-3' and 5'-cgaaaagctgcaaggagatcaatg-3'. A 260 bp genomic DNA fragment located 236 bp upstream of the *Mes3* gene start codon was amplified using the primers 5'-cccatagatatgttaaagttgtg-3' and 5'-ggcagtcacacacacacacacagtc-3'. A 510 bp genomic region approximately 15.2 kb upstream of the *Neu4* gene start codon was amplified using primers 5'-ggacacgacgagctacgacgacacac-3' and 5'-gtggtgaaagttccaccccttgcg-3'. These 1.7 kb, 260 bp, and 510 bp PCR products were cloned directly into pGEM-T Easy Vector (Promega) using the manufacturer's directions, creating pGEM-vndenh, pGEM-Mes3enh, and pGEM-Neu4enh, respectively. pGEM-vndenh, pGEM-Mes3enh, and pGEM-Neu4enh were digested with EcoRI, and EcoRI fragments containing the respective enhancers were isolated and cloned into the unique EcoRI site of -42evelacZCasper (Small et al., 1992), which places the enhancer upstream of the even-skipped minimal promoter driving lacZ reporter expression, creating vndenh.lacZ-Casper, Mes3enh.lacZCasper, and Neu4enh.lacZCasper.

22FPE (Kosman and Small, 1997) provided by S. Small was modified to promote ectopic expression in the *Krüppel* domain. The even-skipped (*eve*) stripe 2 enhancer was removed from 22FPE by NotI digestion and replaced with a ~1.5 kb NotI fragment containing two copies of the *Krüppel* CD1 enhancer identified by Hoch et al. (1990), thereby creating a *Kr-FSF* ectopic expression construct. The *Mes4* open reading frame was PCR amplified from *Drosophila melanogaster* genomic DNA using primers 5'-gctctagatgcgcaatggcagcgaggaactatttg-3' and 5'-gctctagatcaaaagttcatggtccatcaaggaat-3' and cloned into the XbaI site of pBsAsclI, a modified form of pBluescript in which the unique SacI and HincII sites have been converted to Ascl sites, thus creating pBsAsclI-Mes4. An Ascl fragment containing *Mes4* was isolated by Ascl digestion of pBsAsclI-Mes4 and inserted into the unique Ascl site of *Kr-FSF*, creating *Kr-FSF-Mes4*.

P element plasmids were introduced into the *Drosophila* germline using standard methods (Spradling and Rubin, 1982). Plasmid DNA was injected into a strain carrying a mutation in *white* (*yw*<sup>67</sup>), together with the  $\delta$ 2,3 transposase helper plasmid. Multiple independent transformed lines were examined. Appropriately staged embryos were fixed and hybridized with a digoxigenin-UTP-labeled antisense RNA probes as described previously (Tautz and Pfeifle, 1989; Jiang et al. 1991). Antisense probes to previously characterized Dorsal targets (*snail*, *sog*, and *dpp*) have been described (Huang et al., 1997). Antisense probes to other genes were made using ESTs available from Research Genetics (Huntsville, AL); if no EST was available, primers were designed to amplify by PCR 400–1000 bp fragments of coding sequence from either *Drosophila melanogaster* genomic DNA or cDNA (made by reverse transcription of genomic DNA). These fragments were subsequently cloned into pGEM-T Easy Vector, and the resulting plasmids used to generate antisense probes. More information on the generation of probes for specific genes will be made available upon request.

#### Computational Identification of Novel Sequence Motifs in Dorsal Target Enhancers

Genomic DNA fragments (1 kb apiece) that encompass the *twist*, *Mes3*, *rho*, *vnd*, *sog*, and *Neu4* enhancers were compared with a 20 kb control sequence located 5' of the *Abd-B* locus. All possible 7–11 bp sequence motifs were identified using AuiX Biopharma's cis-regulatory bioinformatics package, GeneGrokker version 0.29. Motifs were allowed to include up to two "wild-card" positions denoted by the following symbols: R = A/G, Y = C/T, S = C/G, W = A/T, K = G/T, M = A/C, V = A/C/G, H = A/C/T, D = A/G/T, B = C/G/T, N = A/C/G/T. Motifs were identified that are overrepresented in the enhancer fragments as compared with the control *Abd-B*

sequence. These motifs include Dorsal and Snail recognition sequences (not shown). Novel motifs were also identified, including CACATGT (Figure 5J), which is likely to correspond to a binding site for the bHLH protein, Twist. Two additional motifs were also identified, RGGNCAG (Figure 5K) and GCTGGAA (Figure 5L). The latter sequence motif resembles a 3' Dorsal half-site with extended 3' sequences.

#### Acknowledgments

We thank M. Blanchette, A. Hochheimer, E. Laborier, D. Rio, and R. Tjian for advice and technical assistance with the microarray experiments, and L. Mirels and R. Zinzen for critically reading the manuscript. We also thank R. Zinzen for help with Figure 5M and K. Senger for sharing SELEX data. P. Markstein independently identified the CACATGT motif using a software package, Mermaid, that is under development. We are grateful to H. Ashe for the *Kr-FSF* expression vector and probes and Y. Nibu for providing ESTs. This work was funded by a grant from the NIH (GM46638) to M.L. A.S. is supported by a postdoctoral fellowship from the NIH (GM20352).

Received: May 30, 2002

Revised: October 3, 2002

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#### Accession Numbers

The array data have been deposited in the Gene Expression Omnibus at NCBI (GEO: <http://www.ncbi.nlm.nih.gov/geo>) as series GSE86: GSM2452 (*Toll<sup>mb/m10</sup>* versus *Toll<sup>10b</sup>*), GSM2453 (*pipe* versus *Toll<sup>10b</sup>*), and GSM2454 (*pipe* versus *Toll<sup>mb/m10</sup>*).

The *vnd*, *Mes3*, and *Neu4* embryonic enhancer DNA sequences have been deposited into GenBank with accession numbers BK000635, BK000634, and BK000636, respectively.

# Evolution of TNF Signaling Mechanisms: JNK-Dependent Apoptosis Triggered by Eiger, the *Drosophila* Homolog of the TNF Superfamily

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## Summary

Much of what we know about apoptosis in human cells stems from pioneering genetic studies in the nematode *C. elegans* [1, 2]. However, one important way in which the regulation of mammalian cell death appears to differ from that of its nematode counterpart is in the employment of TNF and TNF receptor superfamilies [3, 4]. No members of these families are present in *C. elegans*, yet TNF factors play prominent roles in mammalian development and disease [1, 3]. Here, we describe the cloning and characterization of Eiger, a unique TNF homolog in *Drosophila*. Like a subset of mammalian TNF proteins, Eiger is a potent inducer of apoptosis. Unlike its mammalian counterparts, however, the apoptotic effect of Eiger does not require the activity of the caspase-8 homolog DREDD, but it completely depends on its ability to activate the JNK pathway. Eiger-induced cell death requires the caspase-9 homolog DRONC and the Apaf-1 homolog DARK. Our results suggest that primordial members of the TNF superfamily can induce cell death indirectly by triggering JNK signaling, which, in turn, causes activation of the apoptosome. A direct mode of action via the apical FADD/caspase-8 pathway may have been coopted by some TNF signaling systems only at subsequent stages of evolution.

## Results and Discussion

Analysis of the *Drosophila* genome sequence reveals a single predicted transcript that encodes a type II membrane protein with structural similarities to members of the TNF superfamily (Figure 1A). We refer to this protein as Eiger, in memory of the numerous mountaineers that have been killed by the Eiger Nordwand, the "wall of death" [5]. The Eiger protein contains a cytoplasmic domain, a transmembrane region between amino acid residues 36 and 62, and an extracellular domain of 353 amino acids (Figure 1A). The C-terminal TNF homology domain (THD) of Eiger shows comparable homology to several human TNF family members (20%–25% identity, Figure 1B). In situ hybridization revealed a weak expres-

sion in imaginal discs with a pronounced pattern in the eye.

Like a subset of human TNF ligands [3], Eiger can induce caspase-dependent apoptosis. Targeted expression of Eiger in the eyes and wings of *Drosophila* causes a severe ablation of these organs (Figures 2A, 2B, and 2E), and Eiger-expressing cell clones are rapidly eliminated (Figures 2J and 2K). Both of these effects can be suppressed by coexpression of the pan-caspase inhibitor p35 [6] (Figures 2C, 2F, 2G, and 2L).

Caspase-8 is the key initiator caspase of death ligand-induced apoptosis in mammals [3]. Upon stimulation by TNF, the adaptor protein FADD recruits and aggregates several molecules of procaspase-8 that mutually cleave and activate each other [7]. Due to the involvement of an extracellular ligand, this pathway has been referred to as the "extrinsic death pathway" [3, 7]. DREDD is the *Drosophila* caspase most similar to caspase-8 and has been shown to physically interact with *Drosophila* FADD [8–11]. Surprisingly, we found that complete removal of DREDD function failed to block Eiger-induced apoptosis (Figure 2I), indicating that Eiger triggers cell death by a DREDD/caspase-8-independent pathway.

Another pathway activated by mammalian TNF death factors is the JNK pathway [3], although its role in inducing apoptosis upon TNF signaling is less well defined [12–15]. JNK signaling in *Drosophila* is reflected by the expression levels of *puckered* (*puc*), a gene encoding a dual-specificity phosphatase that forms a negative feedback loop by downregulating the activity of JNK [16]. We find that high levels of *puc* expression are induced by Eiger (Figures 3F, 3G, 3J, and 3K). Due to its function as a negative regulator of JNK, *Puc* can also be used as a powerful means to repress JNK activity if overexpressed by a constitutive promoter that is no longer dependent on this activity [16, 17]. Coexpression of Eiger and *Puc* completely blocks Eiger activity, strikingly reverting the eye and wing phenotypes to wild-type (Figures 3A, 3B, 3D, and 3E) and blocking Eiger-induced elimination of cell clones (Figures 3L–3N). We observed, however, that forced expression of *Puc* does not prevent all forms of cell death. For example, when tested in the situation of polyglutamine repeat-induced neurodegeneration, which is also caused by apoptosis [18], coexpression of *puc* had no discernible protective effect (Figures 3H and 3I). Taken together, we interpret these results as firm evidence that Eiger induces the JNK signaling pathway and that Eiger-induced apoptosis is critically dependent on JNK activity.

Consistent with our interpretation, we find that several components of the *Drosophila* JNK pathway are rate limiting in mediating or preventing Eiger-induced apoptosis. The removal of one wild-type copy of either *DTRAF1* (encoding the homolog of human TRAF2) [19, 20], *misshapen* (encoding a Ste20 kinase that binds to DTRAF1) [19, 20], or *basket* (encodes *Drosophila* JNK) [20] suppressed Eiger-induced apoptosis (Figures 3P–3R). Conversely, animals heterozygous for a mutation

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A

MTAETLKPFIPTPSANDDGFPAKATSTATAQRRTQLIPLVLGFIGLGLVAILALTIWQTTRVSHLDKELKSLK  
RVVDNLQQRRLGINYLDEFDEFQKEYENALIDYPKKVDGLTDEEDDDDDGGLDSIADDEDDVSYSSVDDV  
GADYEDYDMLNKLNNNAHTGTTPTSETTAEGEGETDSASSASNDNVFDDFTSYNAHKKKQERKRSRIAD  
VRNEEQNIQGNHTELQEKSSNEATSKESPAPLHRRRMHSRHRHLLVRKGESLLSARSEDSPAAHFHLS  
SRRRHQGSMDGYHGDYIGDNDERNNSYQGHFQTRDGVLTVTNTGLYVYVAQICYNNSHDQNGFIVFQGGT  
PFLQCLNTVPTNMPHKVHTCHTSGLIHLERNERIHLKDIHNDRNAVLREGNNRSYFGIFKV

B

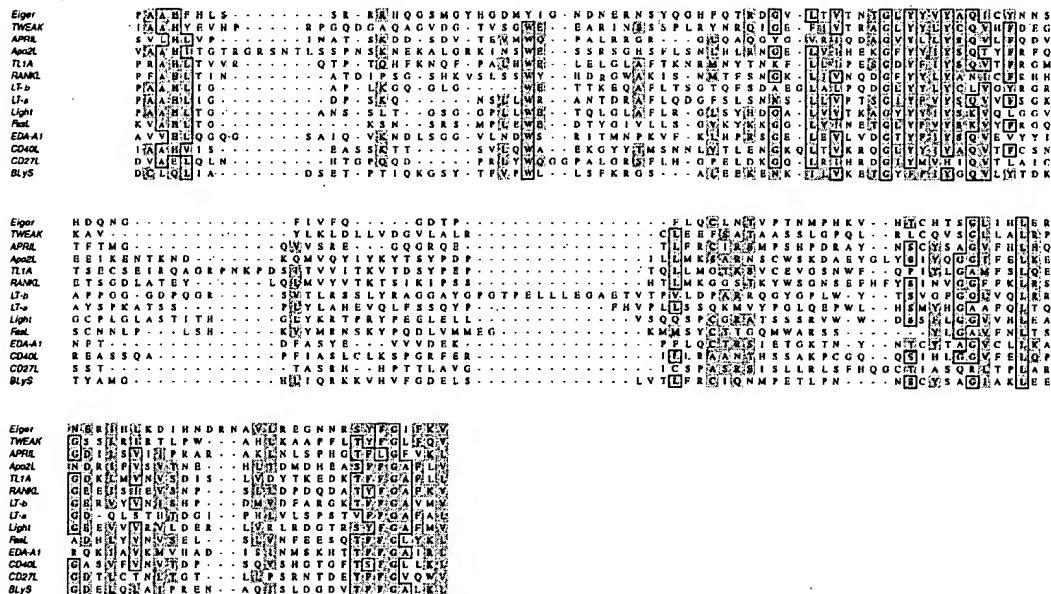


Figure 1. Eiger Is a Member of the TNF Superfamily

(A) The predicted amino acid sequence of Eiger. The predicted transmembrane region (double underlined) and the C-terminal TNF homology domain (THD) (underlined) are indicated.

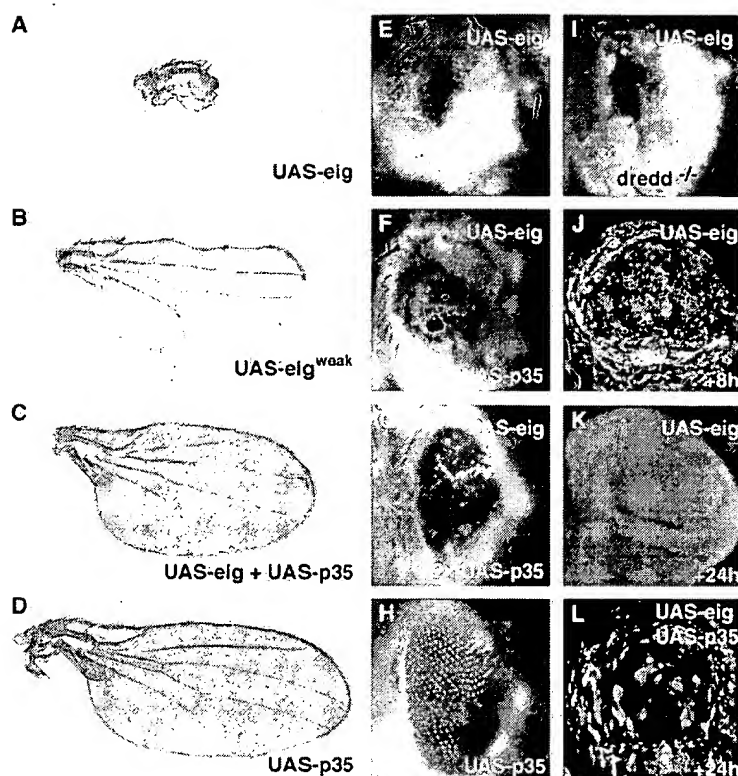
(B) The sequence alignment of the THDs of Eiger and some members of the human TNF ligand family. Identical and homologous residues are boxed and shaded, respectively.

in *puc* [16] display an enhanced phenotype (Figures 3C, S).

The mechanism by which JNK signaling triggers cell death in response to TNF is poorly understood in mammals [12–15] and is unknown in *Drosophila* [17]. We therefore wished to identify the apoptotic machinery responsible for Eiger-induced cell death. Having excluded the caspase-8-like FADD/DREDD branch, we focused on the involvement of caspase-9, which represents another major pathway that leads to apoptosis [7]. The key event for caspase-9 activation is its association with the protein cofactor Apaf-1 to form an active complex referred to as the apoptosome. Since many cell intrinsic insults can trigger this pathway, it has been

termed the “intrinsic death pathway” [7]. Expression of a dominant-negative form of the *Drosophila* caspase-9 homolog DRONC [21–25], comprising only the CARD domain [22], fully blocked Eiger-induced apoptosis in a dose-dependent manner (Figures 4A–4C). Moreover, genetic removal of DARK, the homolog of Apaf-1 [26–28], suppressed Eiger-dependent phenotypes (Figure 4D). These results indicate that the presumptive *Drosophila* apoptosome [25] is essential for the ability of Eiger to induce cell death. In agreement with this conclusion, we found that overexpression of Thread, the *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) [29] blocks Eiger function (Figure 4E). Thread/DIAP1 has been shown to bind DRONC and target it for degradation





**Figure 2. Eiger Induces Cell Death Independent of the Caspase-8 Homolog DREDD**

(A and B) Targeted Eiger expression in the wing. (B) Low levels of *eiger* eliminate wing margin structures, (A) while higher levels of *eiger* ablate the entire organ.

(C) Coexpression of the pan-caspase inhibitor p35 with Eiger suppresses the wing elimination phenotype (compare [A] and [C]).

(D) Targeted expression of p35 in the wing has no discernible phenotype.

(E) Targeted expression of *eiger* in the eye also causes organ ablation.

(F and G) Coexpression of p35 with Eiger suppresses the eye elimination phenotype in a dose-dependent manner (one copy of UAS-p35 in [F], two copies of UAS-p35 in [G]).

(H) Ectopic expression of p35 in the eye causes a subtle ommatidial disorganization [6].

(I) DREDD is not required to mediate Eiger-induced apoptosis. Targeted expression of *eiger* in a *dredd* null mutant background.

(J–L) Clones of cells expressing a UAS-*eiger* transgene are rapidly eliminated by apoptosis. (J) After 8 hr of clone induction, cells expressing *eiger* (marked by GFP expression shown in white) can be seen throughout the wing disc, (K) but they disappear completely after 24 hr. (L) Coexpression of p35 impedes the elimination of *eiger*-expressing clones.

[30]. Most instances of programmed cell death that have been analyzed in *Drosophila* are triggered by, and require, the genes *reaper*, *hid*, or *grim* [31], which encode small proteins that bind to and inactivate IAPs, such as Thread/DIAP1 [32–34]. The removal of one copy of a chromosomal segment that includes the genes *hid*, *grim*, and *reaper* rescues eye ablation (Figure 4F), and Eiger induces a strong transcriptional activation of *hid* (Figures 4G and 4H) and a weak activation of *reaper* (data not shown). These results suggest, therefore, that Eiger/JNK signaling triggers DRONC by inactivating the IAPs via a transcriptional upregulation of *hid*.

Unlike the situation in mammals [3], *Drosophila* TNF appears to activate a linear pathway to induce apoptosis, involving JNK and the apoptosome as landmark components (Figure 4H). Thus, in *Drosophila*, the signaling module formed by FADD and DREDD appears to be dedicated to signaling by the Toll/IMD system [11], whereas, in mammals, this “extrinsic” apoptotic pathway can be activated both by some members of the TNF superfamily [3] and by ligands of Toll-like receptors [35] (Figure 4H). We propose that, at some point during vertebrate evolution, the Toll signaling modules, comprising at least the FADD/caspase-8 branch, but presumably also the NF- $\kappa$ B pathway, were coopted by some members of the TNF/TNFR superfamilies (Figure 4H), expanding their ancestral function of JNK-mediated induction of apoptosis. Other members of the mammalian TNF/TNFR superfamilies may have retained the primordial Eiger mode of signaling with a linear “JNK/apoptosome-only” output.

## Experimental Procedures

### Expression of *eiger*

The *eiger* gene maps to cytological position 46F. A full-length cDNA clone of Eiger was isolated, sequenced (GenBank accession: AF521176), and used to generate a UAS transgene based on pUAST [36]. Two different transformant lines were used, UAS-*eig*, representing the average activity of our transgene insertions, and UAS-*eig*<sup>weak</sup>, which shows only reduced activity. For eye and wing expression, the Gal4 drivers *GMR-Gal4* [37] and *C765-Gal4* [38], respectively, were used.

### Genetic Interactions

For the *dredd* experiments, alleles B118, F64, D44, D55, and L23 were used, all of which are EMS-induced mutations presumed to represent null alleles [8]. All alleles gave the same result when tested in combination with *GMR-Gal4* UAS-*eig*. Flip-out clones expressing *eiger* in discs, alone, and in combination with *puc* or *p35* were generated and marked as described in [17]. *Df(2L)M24F-B* was used to remove one copy of the *Drosophila* *TRAF1* gene. Alleles *msn*<sup>102</sup>, *msn*<sup>172</sup>, *bsk*<sup>1</sup>, *bsk*<sup>2</sup>, and *puc*<sup>ES9</sup> were used to assess the interactions with the *GMR-Gal4* UAS-*eig* genotype. For the identification of downstream components of Eiger-induced apoptosis, the *DARK*<sup>204</sup> allele [28], UAS-*DRONC-CARD* [22], *Df(H99)*, which removes *hid*, *grim*, and *reaper* [39], and an EP insertion upstream of the *DIAP1* locus *thread* (C. Häusermann and K.B., unpublished data) were used.

### Antibodies

Rabbit polyclonal antisera were generated and affinity purified. Staining procedures and other primary and secondary antibodies were used as described in [17].

### Acknowledgments

We would like to thank J. Abrams, N. Bonini, E. Hafen, P. Meier, M. Mlodzik, and B. Lemaitre for fly stocks; V. Dixit, P. Geuking, D.

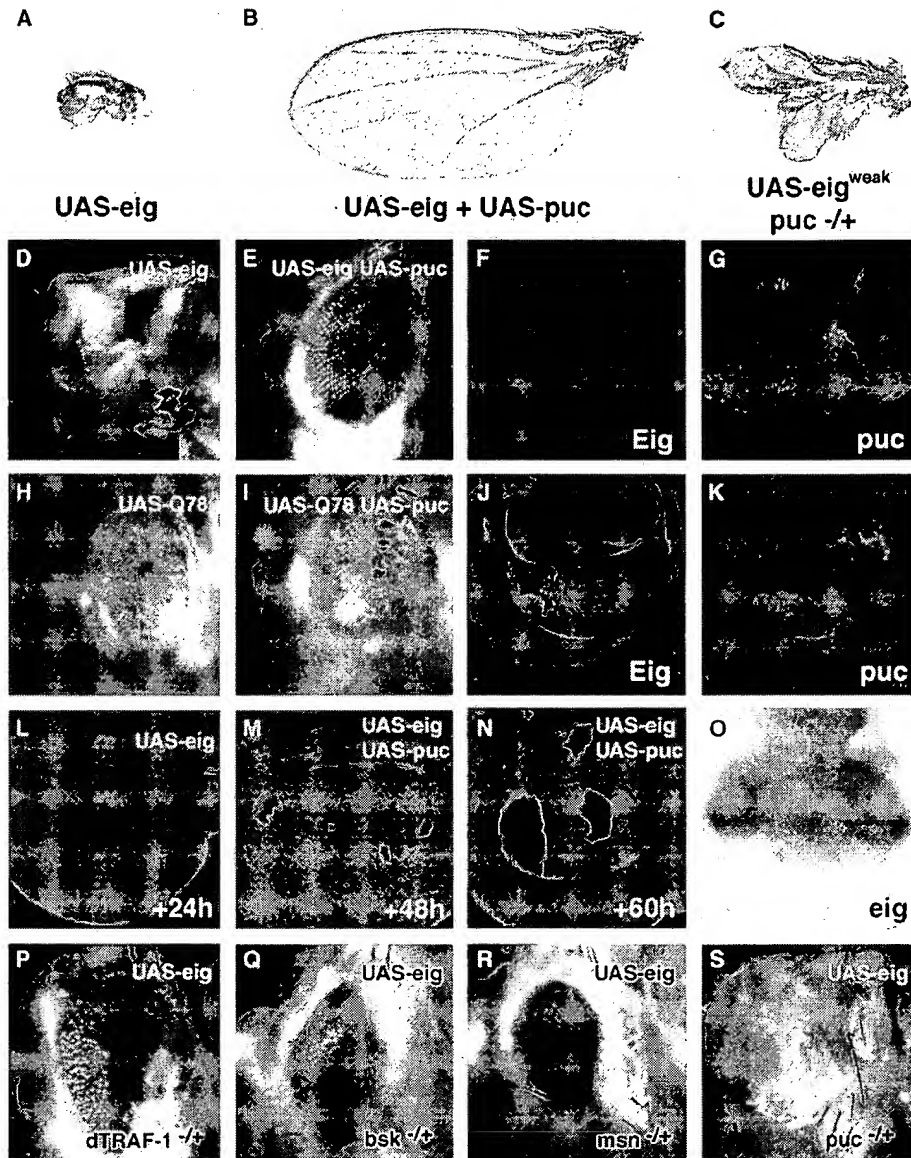


Figure 3. The JNK Pathway Is Essential for Eiger-Induced Apoptosis

(A and B) (A) Organ ablation is completely blocked by (B) inhibiting JNK activity via coexpression of the JNK-phosphatase Puckered (Puc). (C) Elimination of one copy of the endogenous *puc* gene enhances the phenotype of *eiger* overexpression in the wing (compare with Figure 2B). (D and E) (D) The effect of targeted *eiger* expression in the eye can also be blocked by (E) coexpression of *puc*. (F and G) ([F], anti-Eiger staining in green) Targeted expression of *eiger* in developing eye cells activates the JNK pathway, ([G], *puc-lacZ* in red) as monitored by *puc* expression. (H) Expression of the polyglutamine-repeat protein Q-78 in the eye causes a neurodegeneration phenotype [18]. (I) Blocking the JNK pathway by coexpressing *puc* does not block the neurodegeneration phenotype. (J and K) ([J], anti-Eiger staining in green) Targeted expression of *eiger* in the wing disc triggers the JNK pathway, ([K], *puc-lacZ* in red) monitored by *puc* expression. (L–N) Expression of *puc* abolishes Eiger-mediated cell elimination. (M) A total of 48 hr and (N) 60 hr after clone induction, cells coexpressing *eiger* and *puc* are still present (marked by the lack of CD2 expression in green), (L) while cells expressing *eiger* alone have been eliminated from the wing disc after 24 hr. (O) *eiger* mRNA expression detected in the eye disc (posterior to the morphogenetic furrow) by in situ hybridization. (P–R) Eiger-induced eye ablation can be partially suppressed by removing one copy of the (P) *TRAF-1* gene, the (Q) *basket* (*bsk*) gene, or the (R) *misshapen* (*msn*) gene. Conversely, the *UAS-eiger* phenotype is enhanced by eliminating one copy of the *puc* gene (S), similar to what was observed in the wing (C).

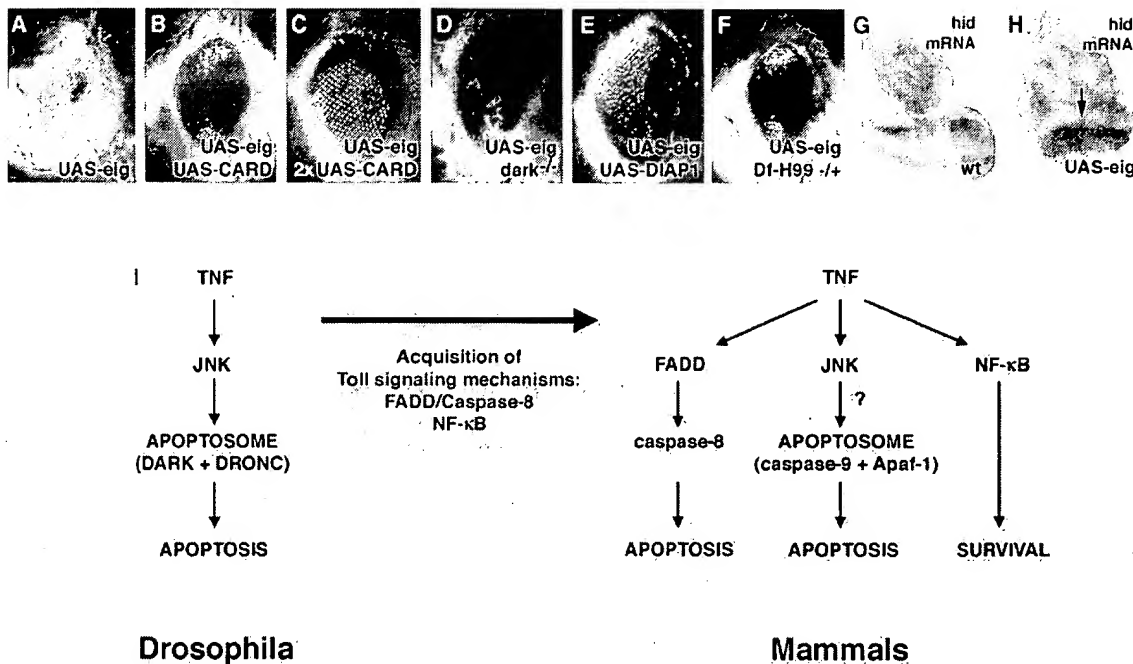


Figure 4. Requirement for the Apoptosome Components DARK and DRONC in Eiger-Induced Apoptosis

- (A) Eye ablation caused by targeted Eiger expression.  
 (B and C) Coexpression of a dominant-negative form of DRONC, containing only the CARD domain, blocks Eiger function in a dose-dependent manner.  
 (D) The *dark<sup>244</sup>/dark<sup>244</sup>* mutant background strongly suppresses the Eiger-induced eye ablation phenotype. DARK is the *Drosophila* homolog of Apaf-1.  
 (E) Coexpression with DIAP1 also blocks Eiger-triggered apoptosis.  
 (F) Eiger-induced eye ablation can be partially suppressed by removing one copy of the genes *hid*, *grim*, and *reaper* using DfH99 [39].  
 (G) Normal expression of *hid* mRNA in the eye disc as revealed by in situ hybridization.  
 (H) Targeted expression of *eiger* upregulates *hid* expression.  
 (I) Proposed model for the evolution of TNF signaling mechanisms (see text).

Nellen, C. von Mering, C. Walser, and F. Wittwer for valuable help; G. Hausman and M. Hengartner for comments on the manuscript; and the Swiss National Science Foundation and the Kanton of Zurich for support.

Received: June 10, 2002  
 Revised: June 14, 2002  
 Accepted: June 14, 2002  
 Published: July 23, 2002

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# Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway

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*Drosophila* provides a powerful genetic model for studying the *in vivo* regulation of cell death. In our large-scale gain-of-function screen, we identified Eiger, the first invertebrate tumor necrosis factor (TNF) superfamily ligand that can induce cell death. Eiger is a type II transmembrane protein with a C-terminal TNF homology domain. It is predominantly expressed in the nervous system. Genetic evidence shows that Eiger induces cell death by activating the *Drosophila* JNK pathway. Although this cell death process is blocked by *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1), it does not require caspase activity. We also show genetically that Eiger is a physiological ligand for the *Drosophila* JNK pathway. Our findings demonstrate that Eiger can initiate cell death through an IAP-sensitive cell death pathway via JNK signaling.

**Keywords:** cell death/*Drosophila*/JNK/TNF

## Introduction

The intrinsic cell death execution mechanisms, which are regulated by the caspases, Apaf-1 and the Bcl-2 family of proteins, are highly conserved throughout evolution (Meier *et al.*, 2000a; Vernooy *et al.*, 2000; Shi, 2001). In *Caenorhabditis elegans*, the regulation of cell death is intrinsic and works through a genetic program. In *Drosophila* and mammals, on the other hand, cell death is largely regulated through extrinsic mechanisms; this is known as social control of cell death (Raff, 1992). Although a number of extrinsic death factors have been identified in vertebrates, until now, none has been reported in invertebrates, despite abundant similarities in the cell death mechanisms between *Drosophila* and vertebrates.

*Drosophila* is a powerful genetic model for studying the *in vivo* role of cell death and its physiological regulation. The intrinsic cell death pathway in flies is stimulated by the *Drosophila* killer proteins, Reaper, Hid and Grim, or a pro-apoptotic Bcl-2 family member, Drob-1/Debcl/dBorg-

1/dBok (Vernooy *et al.*, 2000). Ectopic expression of these cell death trigger proteins in the developing *Drosophila* eye results in a reduced-eye phenotype (Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; Colussi *et al.*, 2000; Igaki *et al.*, 2000). On the other hand, the pro-apoptotic adapter molecules such as *Drosophila* Apaf-1 (Dark) or a *Drosophila* caspase drICE do not cause the reduced eye size (H.Kanuka and M.Miura, unpublished data), suggesting that the small eye phenotype would be generated by an overexpression of the 'apical' cell death triggers, the proteins whose activities are regulated at the transcriptional levels or by the levels of their inhibitory molecules. The reduced-eye phenotype should also be generated by the extrinsic death stimuli such as ligand/receptor-mediated signaling. A *Drosophila in vivo* expression screen has an advantage for identifying the factors that function through such the cell–cell interactions as well as the cell autonomous factors.

To identify novel cell death-inducing factors in *Drosophila*, we screened for genes that had a potential to reduce the eye size when misexpressed in the developing eye. Here, we describe the first invertebrate tumor necrosis factor (TNF) superfamily ligand identified in this screen, Eiger. We provide genetic evidence that Eiger can induce inhibitor-of-apoptosis (IAP)-sensitive cell death through the activation of the *Drosophila* JNK pathway. Genetic analysis of *eiger* mutant flies reveals that Eiger is a physiological ligand for the JNK pathway in the eye disc. Our findings also provide a model for a caspase-independent cell death pathway through the JNK signaling that can be blocked by IAP.

## Results

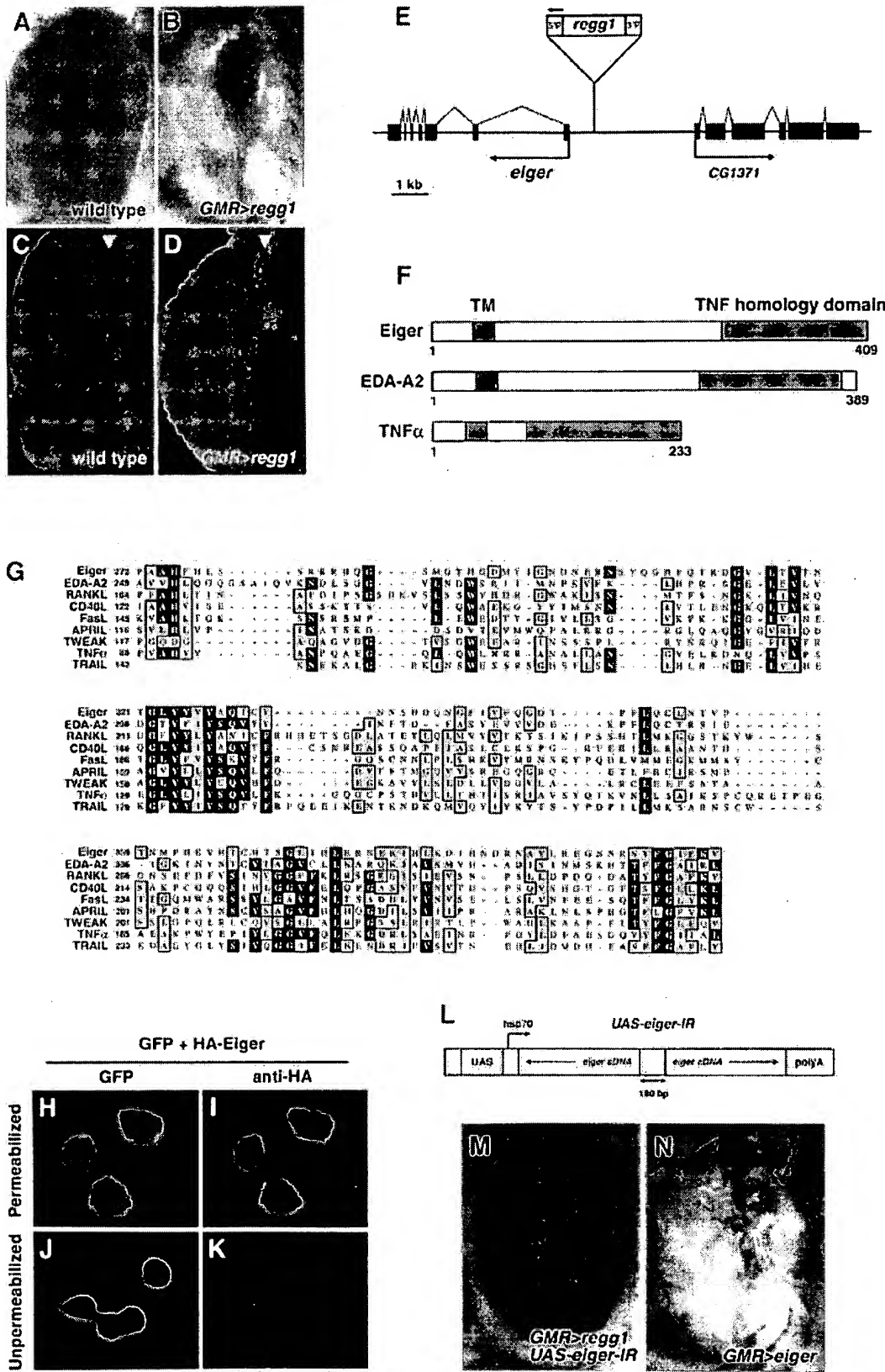
To discover the cell death triggers encoded in the *Drosophila* genome, we conducted a misexpression screen using the GAL4/UAS system. The GS vector is a P element-based gene search vector with UAS enhancers (Toba *et al.*, 1999). We crossed a collection of 5000 lines harboring the GS vector (GS lines) with an eye-specific GAL4 (*GMR-GAL4*) strain to screen for genes that generated the reduced-eye phenotype. Six GS lines resulted in greatly reduced eyes in a GAL4-dependent manner, which we called Regg strains (Regg1–6, for reduced-eye generator with a *GMR-GAL4* driver).

### Identification of a *Drosophila* TNF superfamily ligand, Eiger, as a novel cell death trigger

The F<sub>1</sub> progeny generated by mating the *GMR-GAL4* strain and Regg1 (GS9830) strain (*GMR>regg1<sup>GS9830</sup>*) displayed a strong reduced-eye phenotype (Figure 1B) compared with the wild-type eye (Figure 1A). To assess whether the small eye phenotype of *GMR>regg1<sup>GS9830</sup>* flies was generated by the acceleration of cell death, we

stained eye discs with acridine orange to detect dying cells (Figure 1C and D). Acridine orange is commonly used as a marker for apoptotic cell death in *Drosophila* (Abrams et al., 1993). In *GMR>regg1<sup>GS9830</sup>* discs, numerous

acridine orange-staining cells were observed, indicating that *regg1<sup>GS9830</sup>* induced massive apoptotic cell death, leading to the reduced-eye phenotype. Using the inverse PCR method, we determined the insertion site of the



P element in the *Regg1*<sup>GS9830</sup> strain, and found a predicted gene, *CG12919*, adjacent to the insertion site. The GS9830 strain was generated by inserting the GS6 vector, which has a green fluorescent protein (GFP) trailer (UAS-GFP-SV40 terminator) near the 3'P end and a UAS enhancer near the 5'P end, so that a misexpression of vector-flanking sequence occurs 5'P side only (T.Aigaki, unpublished data). As expected, *CG12919* was the only gene with an elevated expression level in a GAL4-dependent manner (see Supplementary figure S1 available at *The EMBO Journal* Online). We sequenced a *Drosophila* EST clone, LP03784, which included the nucleotide sequence of *CG12919*, and identified the open reading frame (ORF) of a novel gene we named *eiger* (EDA-like cell death trigger; see below) (Figure 1E). *eiger* encoded a protein of 409 amino acids with a C-terminal TNF homology domain and a hydrophobic transmembrane domain, indicating that Eiger was the first *Drosophila* member of the TNF superfamily (Figure 1F). The absence of a signal peptide suggested that Eiger was a type II membrane protein, which is typical of the members of the TNF ligand family. The sequence of the C-terminal TNF domain of Eiger showed highest homology with human EDA-A2 (28% identity), and also showed significant homology with all known TNF superfamily members including RANKL, CD40L, FasL, APRIL, TWEAK, TNF- $\alpha$  and TRAIL (Figure 1G).

To examine whether Eiger was indeed a membrane protein, we transfected S2 cells with expression vectors for an N-terminal hemagglutinin (HA)-tagged Eiger and GFP. In permeabilized cells, cell surface staining was observed in GFP-positive cells by anti-HA immunostaining (Figure 1H and I). When immunostained prior to fixation and permeabilization, however, the staining was not detected (Figure 1K), consistent with Eiger being a type II transmembrane protein with an extracellular C-terminus and a cytoplasmic N-terminus.

To confirm that *eiger* was the gene responsible for the reduced-eye phenotype of *GMR>regg1*<sup>GS9830</sup>, we generated transgenic flies possessing an inverted repeat (IR) expression construct of the *eiger* cDNA (Figure 1L) that would specifically inhibit Eiger expression in a GAL4-dependent manner via a mechanism of RNA interference. This construct worked as a specific inhibitor of Eiger expression in a cell culture system (data not shown). We found that the *Regg1* phenotype was completely rescued by the *eiger*-IR transgene (Figure 1M), demonstrating that *eiger* was indeed the gene responsible for the reduced-eye

phenotype of *GMR>regg1*<sup>GS9830</sup>. Furthermore, ectopic expression of exogenous Eiger in the eye caused a reduced-eye phenotype similar to that of *Regg1*<sup>GS9830</sup> (Figure 1N). Because *regg1*<sup>GS9830</sup> activated *eiger* expression in a GAL4-dependent manner without affecting the expression of adjacent genes (see Supplementary figure S1), we used the *Regg1*<sup>GS9830</sup> strain for further genetic analyses.

### **Overexpression of Eiger results in severe developmental defects that are distinct from the defects caused by Reaper**

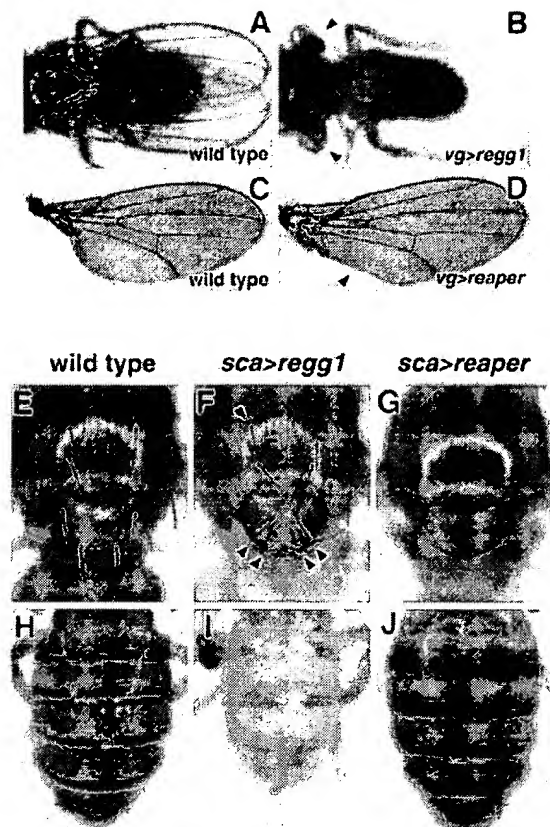
Ectopic expression of Eiger in the eye resulted in a reduced-eye phenotype similar to that of Reaper (White *et al.*, 1996), a *Drosophila* 'intrinsic' cell death trigger (see Figure 3A and E). We further analyzed the effects of Eiger overexpression in other tissues using different GAL4 drivers. When overexpressed in the dorsoventral compartments of the wing discs by a *vg-GAL4* driver, Eiger entirely blocked wing formation (Figure 2B), whereas Reaper induced only a regional defect (Figure 2D). Ectopic expression of Eiger in precursor cells for the external sensory organs by a *sca-GAL4* driver resulted in disorganized macrochaetae in the notum and scutellum (Figure 2F), whereas Reaper induced a complete loss of bristles in these regions (Figure 2G). In the abdomen, on the other hand, *sca>regg1*<sup>GS9830</sup> resulted in a severe developmental defect (Figure 2I), whereas *sca>reaper* caused a loss of bristles in the tergum (Figure 2J). Thus, Eiger has the potential to induce developmental defects distinct from the defects caused by the cell-autonomous killer protein Reaper.

### **Eiger activates an IAP-sensitive cell death pathway that does not require caspase activity**

In mammals, the binding of death ligands such as TNF- $\alpha$  and FasL to their receptors triggers the activation of caspase-8, leading to the subsequent caspase-dependent cell death cascade (Ashkenazi and Dixit, 1998). To assess whether Eiger stimulates similar death signaling in *Drosophila*, we co-expressed Eiger and caspase-inhibitory proteins such as baculovirus P35, *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1) or a dominant-negative form of DRONC (DRONC-DN), a P35-resistant *Drosophila* apical caspase (Meier *et al.*, 2000b). P35 and DRONC-DN exhibited only slight suppressive effects on the Eiger-induced eye phenotype (Figure 3B and D), although they strongly suppressed the Reaper-induced eye ablation, as

**Fig. 1.** Identification of Eiger, a *Drosophila* TNF superfamily protein. (A and B) A misexpression screen identified a GS strain, GS9830 (as *Regg1*), which resulted in a reduced eye when driven by a *GMR-GAL4* driver. Eye phenotypes of wild type (A) and *regg1*<sup>GS9830</sup>/*GMR-GAL4* (B) are shown. (C and D) Acridine orange staining detected numerous dying cells in third-instar larval eye discs of the *regg1*<sup>GS9830</sup>/*GMR-GAL4* strain (D) compared with the wild-type strain (C). Many acridine orange-positive cells were observed behind the morphogenetic furrow (arrowhead), corresponding to the expression domain of the GAL4 driver. (E) A novel ORF encoding Eiger was identified from an EST clone (LP03784) containing the nucleotide sequence of *CG12919*, the expression of which was simulated in a GAL4-dependent manner in the *Regg1*<sup>GS9830</sup> strain. (F) Schematic structures of Eiger, EDA-A2 and human TNF- $\alpha$ . (G) The amino acid sequence of the TNF homology domain of Eiger is aligned with the sequences of human EDA-A2, RANKL, CD40L, FasL, APRIL, TWEAK, TNF- $\alpha$  and TRAIL. Identical and conserved residues are denoted with blocks of black or shading, respectively. The Eiger cDNA sequence has been deposited with DDBJ/EMBL/GenBank (accession No. AB073865). (H–K) Eiger is a type II membrane protein with a cytoplasmic N-terminus. S2 cells were transfected with the expression vectors for HA-Eiger (pUAS-HA-*eiger*) and GFP (pUAS-GFP), together with a driver plasmid, pWAGAL4 (*actin-GAL4*). Twenty-four hours after transfection, cells were immunostained with an anti-HA monoclonal antibody and a Cy3-labeled secondary antibody after (H and I) or prior to (J and K) fixation and permeabilization. (L) Schematic structure of the IR expression construct of *eiger*. Two partial sequences of *eiger* cDNA were cloned as a head-to-head inverted repeat that was separated by a non-palindromic 180 bp linker into the pUAST vector. (M and N) *eiger* is the responsible gene for *Regg1*. The reduced-eye phenotype induced by *regg1*<sup>GS9830</sup> (A) was completely suppressed by *eiger*-IR (M). Overexpression of exogenous Eiger caused a reduced-eye phenotype (N). Genotypes are *regg1*<sup>GS9830</sup>/+; *GMR-GAL4/UAS-eiger*-IR (M) and *UAS-eiger/GMR-GAL4* (N).





**Fig. 2.** Eiger has the potential to induce severe developmental defects that are distinct from the defects caused by Reaper. Eiger or Reaper was ectopically expressed within the dorsoventral boundaries of the wing disc by a *vg-GAL4* driver (B and D). Note that Eiger entirely blocked wing formation (B, arrowheads), whereas Reaper induced only a regional defect (D, arrowhead), compared with wild-type wings (A and C). Ectopic expression of Eiger in precursor cells for the external sensory organs driven by *sca-GAL4* resulted in disorganization of the macrochaetae in the notum and scutellum (F, arrowheads), and a severe developmental defect of the abdomen (I), whereas Reaper induced a complete loss of bristles in these regions (G and J), compared with wild-type fly (E and H). *sca>GAL4* and *UAS-reaper* flies were mated at 18°C, because their progeny, bearing both transgenes, died as pupae when generated at 25°C.

reported previously (White *et al.*, 1996) (Figure 3F and H). These results indicate that Eiger-induced cell death does not require caspase activity differing from the mammalian 'extrinsic' cell death system. In contrast, the co-expression of DIAP1 strongly suppressed the Eiger-induced eye phenotype (Figure 3C), suggesting that DIAP1 could block Eiger-activated death signaling through a mechanism that was independent of caspase inhibition. We also assessed genetically whether endogenous DIAP1 negatively regulates the Eiger-induced phenotype. Whereas heterozygous *diap1* mutant flies exhibit a normal eye (Figure 3I), *GMR>regg1<sup>GS9830</sup>* flies with a half dosage of the *diap1* gene displayed a completely ablated eye phenotype (Figure 3J) compared with the reduced-eye phenotype of *GMR>regg1<sup>GS9830</sup>* flies (Figure 3A), suggesting that DIAP1 is an endogenous inhibitor of Eiger-induced cell death signaling.

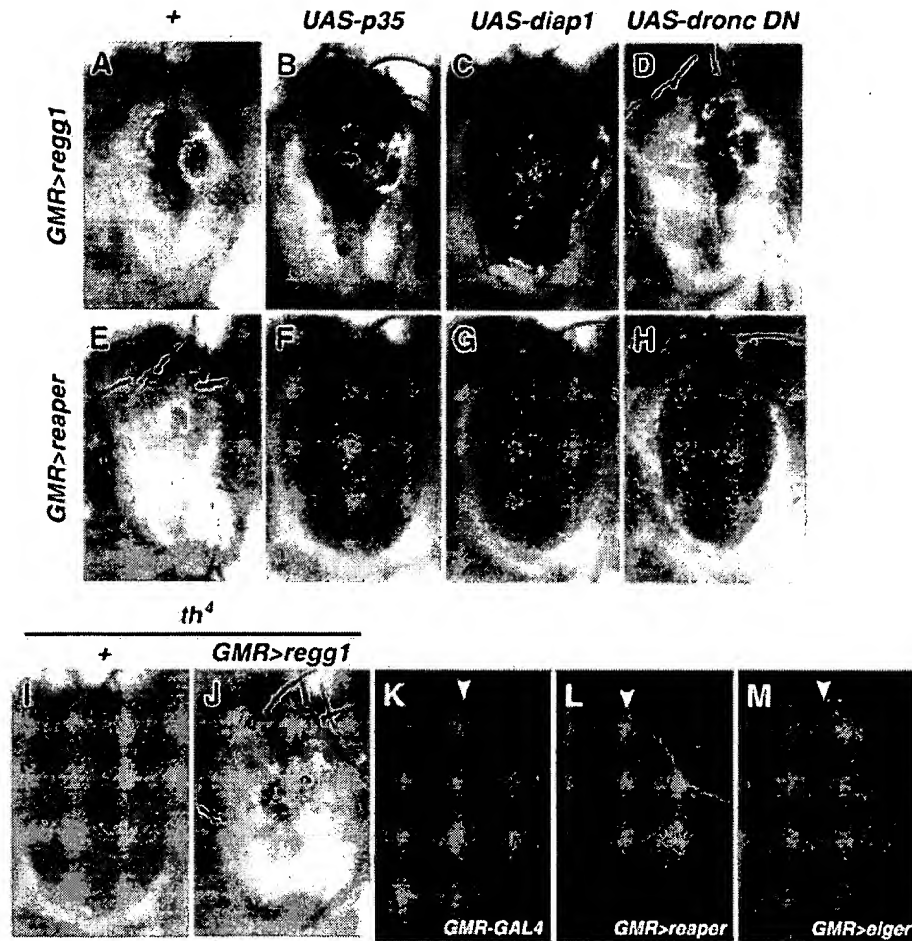
To assess whether Eiger does cause caspase activation, the eye disc was stained with (DME)<sub>2</sub>R, a caspase sub-

strate that contains only an aspartate residue linked to rhodamine-110 (Hug *et al.*, 1999). The eye disc from *GMR>reaper* flies showed a strong rhodamine-110 fluorescence at the region posterior to the morphogenetic furrow (Figure 3L), compared with the eye disc from *GMR-GAL4* flies (Figure 3K). In the *GMR>regg1<sup>GS9830</sup>* eye disc, the fluorescence was detected at lower, but significant, levels in many cells posterior to the furrow (Figure 3M). These data suggest that although caspase activation is not essential for cell death execution, Eiger activates both caspase-dependent and -independent signaling pathways.

### Eiger triggers the *Drosophila* JNK pathway

Many mammalian TNF superfamily proteins activate both the NF-κB and the JNK pathway, and activation of the latter pathway facilitates cell death (Davis, 2000). To examine whether Eiger activates the JNK pathway, we tested the genetic interactions of Eiger with the components of the *Drosophila* JNK cascade (Figure 4A–I). The reduced-eye phenotype induced by Eiger (Figure 4B) was strongly suppressed in a heterozygous mutant of *Drosophila* JNK, *basket (bsk)* (Figure 4C). In addition, overexpression of a dominant-negative form of Bsk almost completely suppressed the eye phenotype (Figure 4D). Moreover, heterozygosity at the *hemipterous (hep)* locus, which encodes *Drosophila* JNKK, suppressed the reduced-eye phenotype much like *bsk* did (Figure 4E), and its hemizygosity (null background) rescued the phenotype almost completely (Figure 4F). Furthermore, the co-expression of a dominant-negative form of dTAK1 (*Drosophila* JNKKK) (Mihaly *et al.*, 2001) also rescued the Eiger-induced phenotype completely (Figure 4G). Misshapen (Msn) is a MAPKKKK that is genetically upstream of the JNK pathway in *Drosophila* (Su *et al.*, 1998). We found that a half dosage of the *msn* gene strongly suppressed the Eiger-induced phenotype (Figure 4H). Heterozygosity of *Drosophila jun*, a target of the JNK pathway, did not show any genetic interaction with Eiger (Figure 4I), raising the possibility that the Eiger-stimulated death-inducing JNK pathway may not require new transcripts that are controlled by Jun.

Puckered (Puc) is a dual-specificity phosphatase, the expression of which is induced by the *Drosophila* JNK pathway to inactivate Bsk, so that *puc* expression can be used to monitor the extent of activation of the JNK pathway (Adachi-Yamada *et al.*, 1999). To confirm that the JNK pathway was actually activated by Eiger, *puc* expression level was assessed in the eye disc of *GMR>regg1<sup>GS9830</sup>* flies using a *puc-LacZ* enhancer-trap allele. The strong induction of *puc-LacZ* was observed in the region posterior to the morphogenetic furrow of the eye disc (Figure 4K) compared with the control eye disc (Figure 4J). Furthermore, western blot analysis with an anti-phospho-JNK antibody revealed that Bsk was phosphorylated by Eiger overexpression (Figure 4L). These genetic and biochemical data led to a model in which Eiger activates Msn, thereby triggering the JNK signaling pathway, sequentially stimulating dTAK1, Hep and Bsk (Figure 4M). We also tested, using RT-PCR analysis, whether Eiger could stimulate the NF-κB pathway; however, no obvious upregulation of the antimicrobial



**Fig. 3.** Eiger activates an IAP-sensitive cell death pathway that does not require caspase activity. (A–H) Genetic interactions of Eiger or Reaper with caspase inhibitory proteins. Genotypes are as follows: *regg1<sup>GS9830</sup>/+*; *GMR-GAL4/+* (A), *regg1<sup>GS9830</sup>/GMR-GAL4*; *UAS-p35/+* (B), *UAS-diap1/+*; *regg1<sup>GS9830</sup>/GMR-GAL4* (C), *regg1<sup>GS9830</sup>/UAS-dronc DN*; *GMR-GAL4/+* (D), *UAS-reaper/GMR-GAL4* (E), *UAS-reaper/GMR-GAL4*; *UAS-p35/+* (F), *UAS-diap1/+*; *UAS-reaper/GMR-GAL4* (G) and *UAS-reaper/UAS-dronc DN*; *GMR-GAL4/+* (H). (I and J) Heterozygosity at the *diap1* locus (*th4*) enhances the Eiger-induced eye phenotype. Whereas flies with a half dosage of the *diap1* gene show a perfectly normal eye (I), the reduced-eye phenotype caused by Eiger overexpression (A) is strongly enhanced by the heterozygosity of *th4* (J), indicating that endogenous DIAP1 negatively regulates the Eiger-stimulated death signal. Genotypes are *th4/+* (I) and *regg1<sup>GS9830</sup>/+*; *GMR-GAL4/th4* (J). (K–M) (DMe)<sub>2</sub>R staining of the eye discs shows that Eiger weakly but significantly causes caspase activation. Caspase activity was detected with rhodamine-110 fluorescence released from (DMe)<sub>2</sub>R. Genotypes are as follows: *GMR-GAL4/+* (K), *UAS-reaper/+*; *GMR-GAL4/+* (L) and *UAS-eiger/+*; *GMR-GAL4/+* (M). Arrowheads indicate the position of the morphogenetic furrow.

peptide genes, the target genes of the *Drosophila* NF- $\kappa$ B pathway, was detected (data not shown).

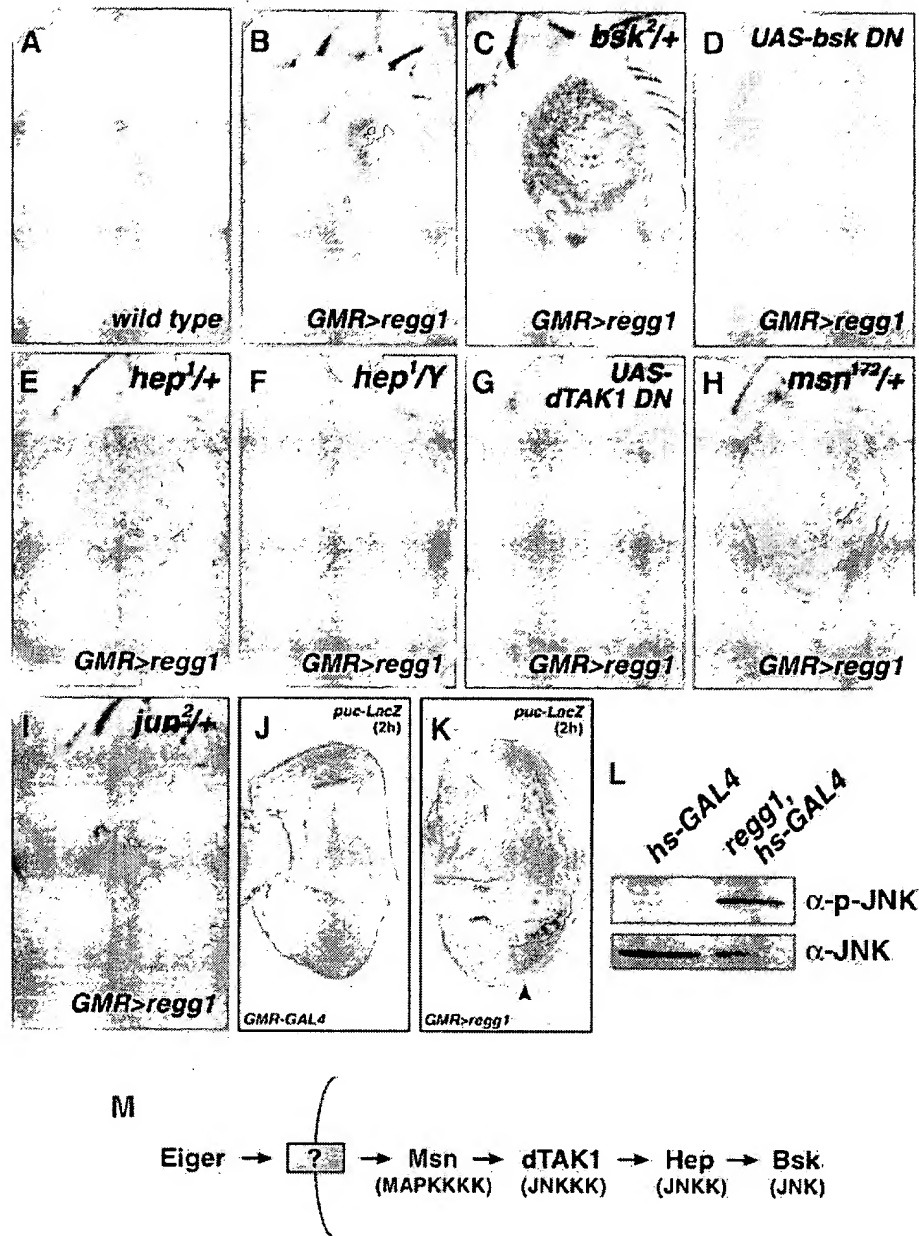
#### **Eiger is predominantly expressed in the nervous system**

The spatial patterns of *eiger* expression during embryogenesis and in larval tissues were analyzed by *in situ* hybridization (Figure 5). Weak signals were detected in pre-blastoderm embryos (stage 1–3), indicating a low level of maternal contribution (Figure 5A). After stage 10, *eiger* transcripts were predominantly detected in the nervous system of the embryos (Figure 5B, C and G–J). In the third-instar larva, *eiger* was strongly expressed in the brain hemispheres (Figure 5K) and at the morphogenetic furrow in the eye disc (Figure 5M); it was also expressed at significant levels in many cells posterior to the furrow. Double staining of the eye disc with the *eiger* RNA probe and an anti-ELAV antibody, which recognizes terminally

differentiated neuronal nuclei, revealed that *eiger* was strongly expressed in the proliferating cells at the furrow (Figure 5O and P). RT-PCR analysis demonstrated that *eiger* mRNA was expressed at all stages of *Drosophila* development (data not shown).

#### **Eiger is a physiological ligand for the *Drosophila* JNK pathway**

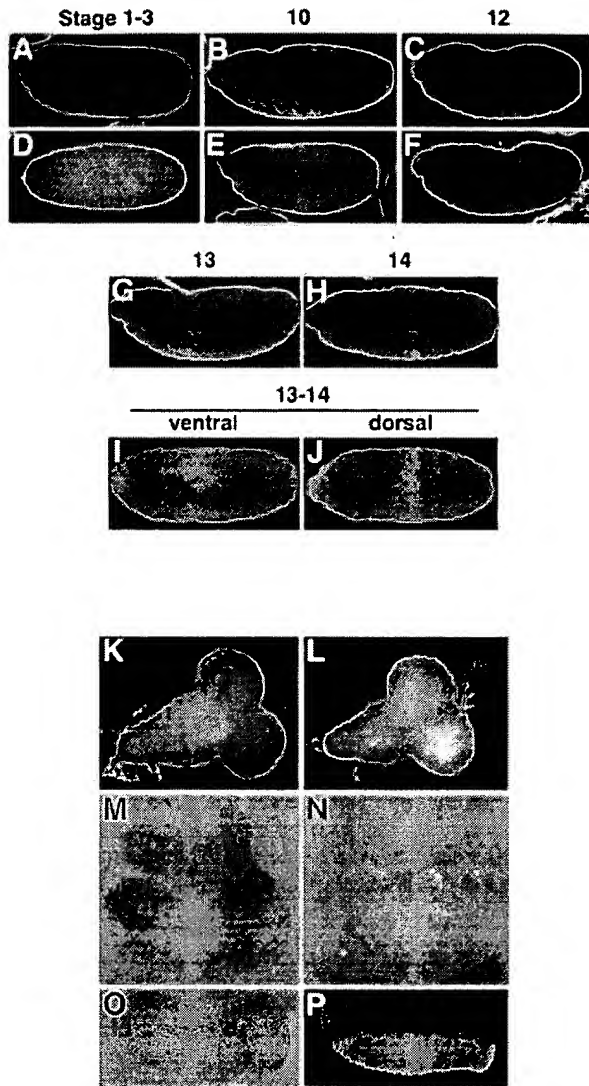
To analyze the physiological role of Eiger, we generated a loss-of-function mutant for *eiger* by imprecise excision of the P element in the *Regg1<sup>GS9830</sup>* strain. We isolated nine mutants harboring deletions that removed parts of the *eiger* genomic sequences, but leaving right sequences downstream of the P element intact (Figure 6A and B). *egr1* and *egr3* mutants were homozygous viable with greatly reduced *eiger* expressions (Figure 6C and E). In the eye disc of the *puc-LacZ* enhancer-trap line, endogenous JNK activity could be detected in the region posterior to



**Fig. 4.** Eiger induces a reduced-eye phenotype through the activation of the JNK pathway. (A–I) *regg1<sup>GS9830</sup>* genetically interacts with components of the *Drosophila* JNK signaling pathway. The Eiger-induced phenotype is suppressed by heterozygosity at the *bsk*, *hep* or *msn* locus (C, E and H, respectively). Hemizygosity at the *hep* locus almost completely suppresses the phenotype (F). Co-expression of a dominant-negative form of Bsk or dTAK1 completely inhibits the Eiger-induced eye phenotype (D and G). Heterozygosity at the *Drosophila jun* locus does not suppress the eye phenotype (I). Genotypes are as follows: wild type (A), *regg1<sup>GS9830</sup>/GMR-GAL4; TM3,Sb/+* (B), *regg1<sup>GS9830</sup>/bsk<sup>2</sup>; GMR-GAL4/+* (C), *regg1<sup>GS9830</sup>/GMR-GAL4; UAS-bsk DN/+* (D), *hep<sup>1/+</sup>; regg1<sup>GS9830</sup>/+; GMR-GAL4/+* (E), *hep<sup>1/Y</sup>; regg1<sup>GS9830</sup>/+; GMR-GAL4/+* (F), *regg1<sup>GS9830</sup>/UAS-dTAK1 DN; GMR-GAL4/+* (G), *regg1<sup>GS9830</sup>/+; GMR-GAL4/msn<sup>172</sup>* (H) and *regg1<sup>GS9830</sup>/jun<sup>2</sup>; GMR-GAL4/+* (I). (J and K) Overexpression of Eiger activates the JNK pathway. The JNK activation was monitored in *GMR-GAL4/+* (J) and *regg1<sup>GS9830</sup>/+; GMR-GAL4/+* (K) background eye disc by *puc-LacZ* expression. X-gal staining (2 h) of the eye disc shows dramatic activation of the JNK pathway in the region posterior to the morphogenetic furrow (K, arrowhead), where Eiger is overexpressed. (L) Eiger stimulates the phosphorylation of Bsk *in vivo*. *hs-GAL4/+* or *regg1<sup>GS9830</sup>/+; hs-GAL4/+* larvae were heat shocked at 37°C for 45 min and cultured at 25°C for a further 4 h, and then subjected to western blot analysis with an anti-JNK or an anti-phospho-JNK antibody. (M) A model for the JNK signaling triggered by Eiger.

the morphogenetic furrow by long-time (20 h) X-gal staining (Figure 6F, arrowhead). We found that the *puc-LacZ* expression was dramatically reduced in the eye disc of the *eiger* mutant (Figure 6G). Notably, the JNK activities at the marginal region of the eye disc, where

endogenous *eiger* expression was not detected (Figures 5M and 6D), were not affected in the *eiger* mutant (Figure 6F and G, arrows). These findings indicate that Eiger functions as a physiological ligand for the *Drosophila* JNK pathway.



**Fig. 5.** *eiger* is predominantly expressed in the nervous system. (A–J) Whole-mount *in situ* hybridization of wild-type embryos at various stages of development using *eiger* antisense (A–C and G–H) or sense (D–F) RNA probes. A pre-blastoderm embryo showed low levels of *eiger* expression (A). After germ band extension, strong staining was evident in the nervous system (B, C and G–J). (K–N) *In situ* analysis of *eiger* expression in third-instar larval brains (K and L) and eye discs (M and N) using *eiger* antisense (K and M) or sense (L and N) RNA probes. A high level of *eiger* expression was evident in the brain hemispheres (K). In the eye disc, stronger staining was detected at the region posterior to the morphogenetic furrow (M). Double staining of the eye discs with the *eiger* RNA probe (O) and the anti-ELAV antibody (P) revealed that *eiger* was strongly expressed in the proliferating cells at the furrow.

## Discussion

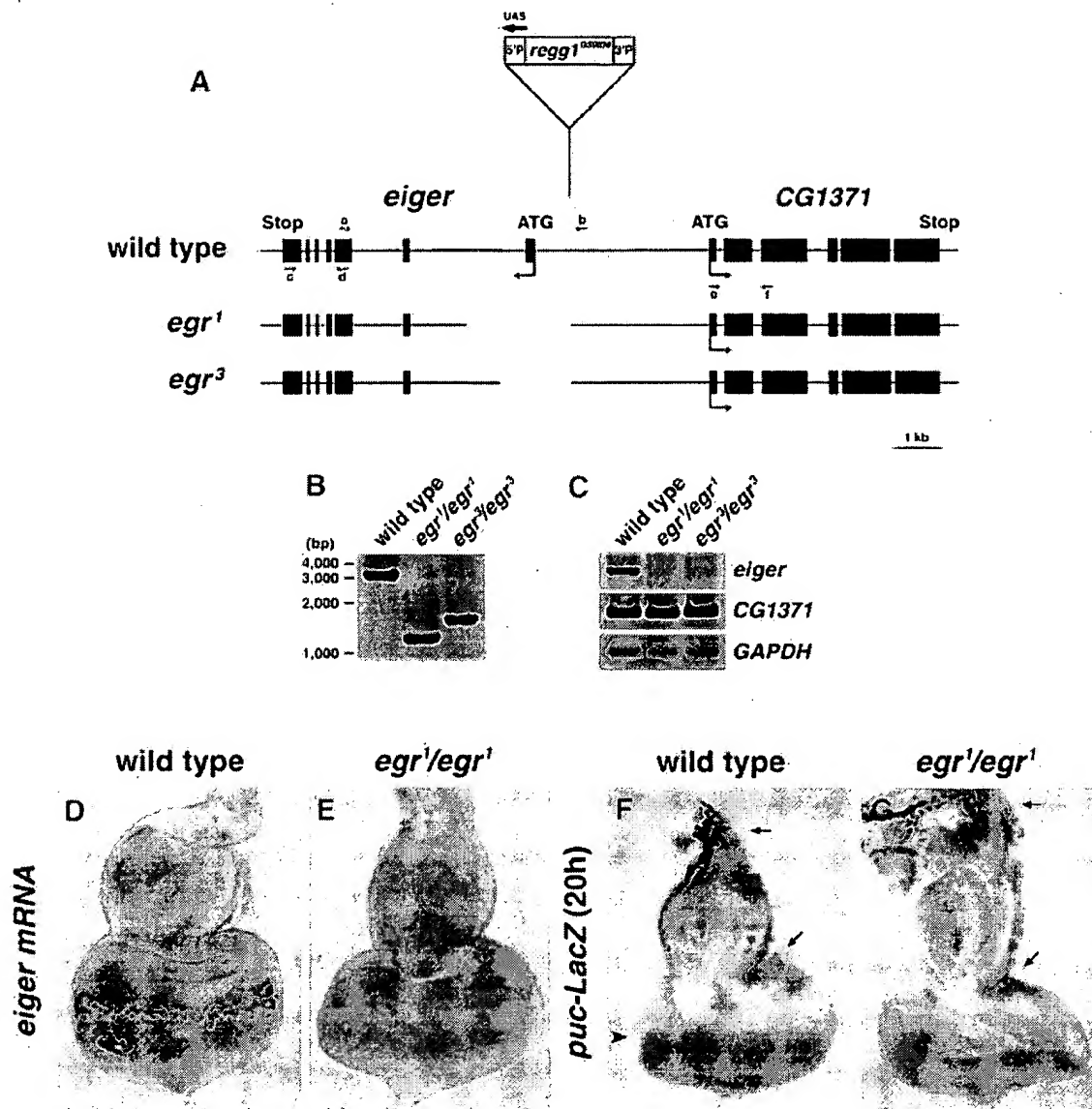
In our *in vivo* expression screen, we identified Eiger as a novel cell death trigger molecule in *Drosophila*. The structure and function of Eiger suggest that the extrinsic cell death-inducing mechanism might be evolutionarily conserved in *Drosophila*. Genetic evidence reveals that caspase activation is not essential to execute Eiger-induced cell death. The *Drosophila* extrinsic cell death system might predominantly utilize the caspase-independent pathway, in contrast to the intrinsic cell death system,

which is regulated by Reaper, Hid and Grim, and depends completely on caspase activation. Although caspases do take part in the apoptotic effects of most of the mammalian TNF ligand/receptor superfamily members studied so far (Ashkenazi and Dixit, 1998; Locksley *et al.*, 2001), there is accumulating evidence that they can also kill the cells in the absence of caspases (Holler *et al.*, 2000; Matsumura *et al.*, 2000; Denecker *et al.*, 2001).

The genetic data presented here clearly show that the Eiger-induced small eye phenotype depends strongly on the JNK signaling pathway. In mammals, it has been demonstrated that the JNK pathway is essential for the execution of stress-induced cell death. JNK3, a JNK isoform that is selectively expressed in the nervous system, is required for neuronal cell death caused by excitotoxic stress (Yang *et al.*, 1997). Embryonic fibroblasts from mouse deficient for both JNK1 and JNK2 are resistant to UV-stimulated apoptosis (Tournier *et al.*, 2000). Whitfield *et al.* (2001) have shown that Bim acts downstream of the JNK pathway in NGF-deprivation-induced neuronal cell death. One possible downstream mechanism of the JNK pathway to induce cell death may be transcriptional upregulation of Bim. However, our results suggest the possibility that Eiger-induced cell death signaling may be independent of downstream *jun* expression, similar to the observation that the effect of UV to cause cell death does not require new gene expression (Tournier *et al.*, 2000). The JNK signaling also mediates heat shock-induced cell death, the execution of which is caspase independent (Gabai *et al.*, 2000). Furthermore, overexpression of the EDA receptor or TAJ/TROY, a member of the TNF receptor superfamily that exhibits extensive homology to the EDA receptor, results in the activation of the JNK pathway and caspase-independent cell death (Eby *et al.*, 2000; Kumar *et al.*, 2001). In some cases, JNK-induced cell death is mediated by the release of mitochondrial apoptogenic factors (Tournier *et al.*, 2000). Recently, it has been shown that cancer cell death induced by TRAIL, a mammalian TNF superfamily ligand, requires mitochondrial release of Smac (Deng *et al.*, 2002). One possible mechanism of Eiger-induced cell death may be JNK-mediated release of mitochondrial caspase-independent cell death factors. In fact, the *Drosophila* genome also encodes homologs of such molecules: AIF, endo G and HtrA2.

One important feature of Eiger-stimulating cell death signaling is that it can be blocked by DIAP1. It is well understood that IAP family proteins suppress cell death through direct inhibition of caspases (Deveraux and Reed, 1999). Our observations suggest a potential mechanism of IAP that can inhibit caspase-independent cell death. It has been reported that *Xenopus* cell death induced by TAK1 and TAB1, an activator for TAK1, is blocked by X-chromosome-linked IAP (XIAP) (Yamaguchi *et al.*, 1999). More recently, it has been shown that XIAP attenuates TNF- $\alpha$ -mediated JNK activation in HeLa cells and *RelA*<sup>-/-</sup> fibroblasts (Tang *et al.*, 2001). These findings and our data led to a model in *Drosophila* in which DIAP1 regulates caspase-dependent and -independent cell death pathways by blocking both the caspases and the JNK signaling.

Our loss-of-function study demonstrates that Eiger is a physiological trigger for the JNK pathway in the



**Fig. 6.** Eiger is a physiological ligand for the JNK pathway. (A) Diagram of the *eiger* genomic locus and location of the end points of excision mutations in *egr1* and *egr3* mutant alleles. Exon sequences are shown by closed boxes. (B) PCR analysis of genomic DNA from wild-type and homozygous *eiger* mutant adult flies using primers a and b [indicated in (A)]. The end points of genomic deletions in *eiger* mutants were determined by sequencing of the PCR products. (C) RT-PCR analysis of cDNA from wild-type and homozygous *eiger* mutant adult flies using primers c and d or e and f [indicated in (A)] to determine the expression levels of *eiger* and *CG1371*, respectively. *GAPDH* expression was determined as an internal control. (D and E) *In situ* analysis of *eiger* expression in the eye disc from wild-type (D) and *egr1/egr1* (E) larvae. (F and G) The JNK activity was monitored in wild-type (F) and *egr1/egr1* (G) background eye discs by *puc-LacZ* expression. Long-time (20 h) X-gal staining was able to detect the endogenous JNK activity in the region posterior to the morphogenetic furrow (F, arrowhead). The JNK activity was dramatically reduced in the *eiger* mutant (G). Note that the JNK activities in the disc margin, where *eiger* is not expressed even in the wild-type disc, are not affected in the *eiger* mutant (F and G).

eye disc. We also show by genetic interaction assays that Eiger-stimulating cell death signaling is mediated by Msn, dTAK1, Hep and Bsk. Although dominant-negative dTAK1 completely suppressed the Eiger-induced phenotype in our experiments, it is also possible that many components of MAP kinase pathways expressed as 'dominant negatives' can have a gain-of-function inhibitory activity. In fact, the immune response phenotype of dTAK1 mutants seems to be inconsistent with the idea that dTAK1 participates in the Eiger pathway (Vidal *et al.*, 2001). Another possible JNK family member to

mediate Eiger signaling is Slipper (Stronach and Perrimon, 2002). Previous genetic studies in *Drosophila* have revealed that the JNK signaling pathway regulates epithelial morphogenesis during the process of embryonic dorsal closure, and also participates in the control of planar polarity in several tissues (Noselli and Agnes, 1999). It has also been reported that the JNK signaling regulates cell death to maintain normal morphogenesis of the wing (Adachi-Yamada *et al.*, 1999). Eiger might function as a JNK-dependent cell death regulator to facilitate normal morphogenesis of the eye. Further analysis of *eiger* mutant

flies would dissect the physiological role of Eiger in neural development.

In mammals, members of the TNF superfamily play crucial roles in the regulation of infections, inflammation, autoimmune diseases and tissue homeostasis (Locksley *et al.*, 2001). The TNF superfamily ligands bind to their respective receptors leading to the activation of diverse signaling pathways, including the caspase cascade, NF- $\kappa$ B, or MAPKs such as JNK or ERK. Thus, TNF-related ligands can trigger either the extrinsic cell death execution, differentiation or proliferation. Although over-expression of Eiger can strongly induce cell death in the *Drosophila* compound eye, we can not exclude the possibility that Eiger-stimulated signaling may contribute to cellular events other than cell death execution. In fact, the amino acid sequence of Eiger showed the highest homology (19%) with EDA, a human TNF superfamily ligand, the mutation of which causes impaired ectodermal development. *eiger* is predominantly expressed in the nervous system, whereas most mammalian TNF/TNF receptor superfamily proteins are expressed in the immune system, raising the possibility that Eiger might regulate proliferation of neural progenitor cells such as TNF- $\alpha$  (Arnett *et al.*, 2001) to maintain normal development of the nervous system. The *Drosophila* genome has a gene encoding a candidate Eiger receptor with a TNF receptor homology domain and a transmembrane domain. In addition, the *Drosophila* genome also encodes genes for mediating factors such as TNF-receptor-associated factors (TRAFs) (Liu *et al.*, 1999; Zapata *et al.*, 2000; Shen *et al.*, 2001), FADD (Hu and Yang, 2000) and RIP (IMD) (Georgel *et al.*, 2001), all of which may play a role in Eiger/Eiger receptor signaling. Further genetic study of Eiger and its receptor should help elucidate the universal role of TNF/TNF receptor superfamily proteins in normal development, as well as in some pathophysiological conditions.

## Materials and methods

### Molecular cloning and expression constructs

The genomic DNA surrounding *regg*<sup>GS9830</sup> was cloned by an inverse PCR method (<http://www.fruitfly.org/about/methods/inverse.pcr.html>) and sequenced. A *Drosophila* EST clone LP03784 was sequenced, and *eiger* cDNA was amplified by PCR, cloned into the *Eco*RI-*Xba*I sites of the pUAST vector to generate pUAS-*eiger* using the following PCR primers: 5'-AAAGAATTCACCATGACTGCCGAGACCCTCAA-3' and 5'-AAATCTAGATTACACCTTGAAGATGCCAAA-3'. A head-to-head inverted repeat construct, pUAS-*eiger*-IR, was generated by inserting the *eiger* cDNA fragment (nucleotides 177–1230) into the *Eco*RI site of pUAS-*eiger*. An expression construct for HA-tagged Eiger was generated by PCR.

### Histology, in situ hybridization and immunohistochemistry

Acridine orange staining was performed as described by Wolff and Ready (1991). (DMe)<sub>2</sub>R (Calbiochem) staining of third-instar larval eye disc was performed at final concentration of 50  $\mu$ M in phosphate-buffered saline for 15 min at 37°C. *In situ* hybridization with digoxigenin-labeled *eiger* RNA probe was carried out as described by White *et al.* (1996). Anti-ELAV staining of the eye disc was performed using a monoclonal antibody, Elav-9F8A9 (1:10), and a Cy3-labeled anti-mouse IgG secondary antibody as described previously (Kanuka *et al.*, 1999). Immunostaining of S2 cells was performed using an anti-HA monoclonal antibody (1:200) (12CA5; Boehringer Mannheim). The immunoreaction was carried out before or after fixation (4% PFA) and permeabilization (0.1% Triton X-100).

### RT-PCR

For RT-PCR analysis of the genes surrounding *regg*<sup>GS9830</sup>, total RNA was prepared from *regg*<sup>GS9830/+</sup>; *hs-GAL4/+* flies using Trizol (Gibco-BRL) 3 h after treatment with or without heat shock (twice at 37°C for 30 min with a 30 min interval), reverse transcribed, and subjected to PCR analysis (26 cycles) using the following primers: CG12919 (*eiger*), 5'-ATGACTGCCGAGACCCTCAAGCCG-3' and 5'-TTACACCTTGAAGATGCCAAAGTAG-3'; CG1371, 5'-GAAGTCGTGCGGCTGCGGTGGATTG-3' and 5'-GATTTGTCCAAGTGGGAGAAGCAG-3'; CG2269, 5'-ATGGCAGCAATGGCCAACCGATTG-3' and 5'-CTCCTTGGGCGTAACATTACCCGC-3'; *cde2rk*, 5'-ATGTCCAGCTTAAGTCAAACGATG-3' and 5'-GCAGCAGCTACCAAGAATGCAGC-3'; *GAL4*, 5'-GCCAATTTAATCAAAGTGGGAATA-3' and 5'-GTTTGGTGGGTATCTTCATCATC-3'; and *GAPDH*, 5'-CCACTGCCGAGGAGGTCAACTA-3' and 5'-GCTCAGGTGATTGCGTATGCA-3'. For antimicrobial peptide genes, cDNA prepared from heat-shocked *hs-GAL4/+* or *regg*<sup>GS9830/+</sup>; *hs-GAL4/+* flies was subjected to PCR analysis (26 cycles) using primers specific for *dipterican*, *cecropin A*, *defensin*, *attacin* and *drosomycin* (Kim *et al.*, 2000).

### Cell culture, transfection and western blotting

S2 cells were cultured and transfected as described previously (Igaki *et al.*, 2000). For western blotting, an anti-JNK polyclonal antibody (1:300; Santa Cruz) and an anti-phospho-JNK (p-JNK) polyclonal antibody (1:300; Promega) were used.

### Fly stocks

Fly culture and crosses were carried out at 25°C, unless otherwise stated. *Canton-S* or *white*<sup>1118</sup> was used as a wild-type strain. *GMR-GAL4* virgins were crossed to males from the collection of GS strains (Toba *et al.*, 1999). *UAS-p35*, *UAS-diap1*, *UAS-dronc* DN, *UAS-bsk* DN, *UAS-dTAK* DN (*K46R*) strains, *th<sup>4</sup>*, *bsk<sup>2</sup>*, *hep<sup>1</sup>*, *msn<sup>172</sup>* and *jun<sup>2</sup>* mutant strains, and a *puc<sup>E69</sup>* enhancer-trap line were used for genetic interaction assays. We also used *hs-GAL4*, *sca-GAL4* and *vg-GAL4* strains. *UAS-eiger* and *UAS-eiger-IR* transgenic flies were generated by general P element-mediated transformation.

### Generation of eiger mutants

The *eiger* mutant alleles were isolated in a screen for imprecise excisions of the P element in the *Regg*<sup>GS9830</sup> strain. The mini-*white* (*w<sup>+</sup>*)-marked P element was mobilized by the  $\Delta 2-3$  transposase source. Two hundred and four fly lines in which the P element sequences had been excised were identified by their white eye color. Nine imprecise excisions, which removed parts of the *eiger* genomic sequences without affecting right sequences downstream of the P element, were identified by PCR analysis using primers corresponding to genomic sequences flanking the P element insertion site. Seven homozygous viable mutant lines were obtained with significantly decreased *eiger* expression.

### Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

## Acknowledgements

We are grateful to Naoko Tokushige, Tetsuo Hiratou and Ryoko Akai for technical support, Ryosuke Takahashi for critical reading of the manuscript, and Hideyuki Okano for invaluable advice. We also thank Jozsef Mihaly, Marek Mlodzik, Bruce Hay, Gerald Rubin, Herman Steller, John Nambu, Takashi Adachi-Yamada, Makoto Nakamura and the Bloomington Stock Center for fly stocks, Yasushi Hiromi for the pWAGAL4 plasmid, and John Gurdon and Ryusuke Niwa for the pUAS-*GFP* plasmid. We are grateful to Yasuo Uchiyama for encouragement. This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, Culture and Technology (to M.M.). E.K. is a research fellow of the Junior Research Associate Program, RIKEN. H.K. is a research fellow of the Special Postdoctoral Researchers Program, RIKEN. T.I. and H.K. are research fellows of the Japan Society for the Promotion of Science.

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Received March 22, 2002; revised and accepted April 29, 2002



## Targeted gene expression as a means of altering cell fates and generating dominant phenotypes

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### SUMMARY

We have designed a system for targeted gene expression that allows the selective activation of any cloned gene in a wide variety of tissue- and cell-specific patterns. The gene encoding the yeast transcriptional activator GAL4 is inserted randomly into the *Drosophila* genome to drive GAL4 expression from one of a diverse array of genomic enhancers. It is then possible to introduce a gene containing GAL4 binding sites within its promoter, to activate it in those cells where GAL4 is expressed, and to observe the effect of this directed misexpression on development. We have used GAL4-directed transcription to expand the domain of embryonic expression of the homeobox protein *even-skipped*. We show that

*even-skipped* represses *wingless* and transforms cells that would normally secrete naked cuticle into denticle secreting cells. The GAL4 system can thus be used to study regulatory interactions during embryonic development. In adults, targeted expression can be used to generate dominant phenotypes for use in genetic screens. We have directed expression of an activated form of the Dras2 protein, resulting in dominant eye and wing defects that can be used in screens to identify other members of the Dras2 signal transduction pathway.

Key words: targeted expression, enhancer trap, GAL4, cell fate

### INTRODUCTION

The ability to express a gene in a directed fashion is a useful means of analyzing its role in development. A switch in cell fate can often be induced by ectopic expression of a gene. The ability to alter transcription patterns at will would permit direct testing of models for cell fate determination. For example, misexpression of a ligand can be used to assay whether a receptor is restricted to specific cells or is present, and competent to respond to ligand-induced activation, in every cell of a particular tissue (such as the ability of cells expressing the *sevenless* receptor tyrosine kinase to respond to the presence of the ligand, *boss*; Van Vactor et al., 1991). A gene encoding a putative transcriptional activator (or repressor) can be expressed in a different cell, or at a different time of development, and its effect on the subsequent expression of a target gene assayed. In this way it is possible to determine if the activator is both necessary and sufficient for transcription of the target. Such an analysis is particularly useful where a protein acts as an activator in one context and as a repressor in another (as may be the case for the zinc-finger protein *Kruppel*; Sauer and Jackle, 1991).

To date, two methods have been employed most widely to manipulate gene expression. The first is to drive expression of a gene from a heat shock promoter. The gene can then be turned on at a specific point in development by

heat shocking the transgenic animal (Struhl, 1985; Schneuwly et al., 1987; Ish-Horowicz and Pinchin, 1987; Ish-Horowicz et al., 1989; Gonzales-Reyes and Morata, 1990; Blochlinger et al., 1991; Steingrimsson et al., 1991). An advantage of the heat shock method is that it permits inducible expression; several disadvantages are that ectopic expression is ubiquitous, that basal levels of expression are observed from heat shock promoters, and that heat shock itself can induce phenocopies (Petersen and Mitchell, 1987; Petersen, 1990; Yost et al., 1990). The second technique is to drive expression of a gene using the transcriptional regulatory sequences from a defined promoter (Zuker et al., 1988; Parkhurst et al., 1990; Parkhurst and Ish-Horowicz, 1991). The use of tissue-specific promoters allows transcription to be restricted to a defined subset of cells, but is limited by the availability of cloned and characterized promoters that can direct expression in the desired pattern. Furthermore, if the gene product to be expressed is toxic to the organism, it is impossible to establish stable transgenic lines carrying the chimeric gene.

Here we describe a method for directing gene expression in *Drosophila* that overcomes these difficulties (Fig. 1). First, the system allows the rapid generation of individual strains in which ectopic expression of the gene of interest (the target gene) can be directed to different tissues or cell types. Second, the method separates the target gene from its transcriptional activator in two distinct transgenic lines.

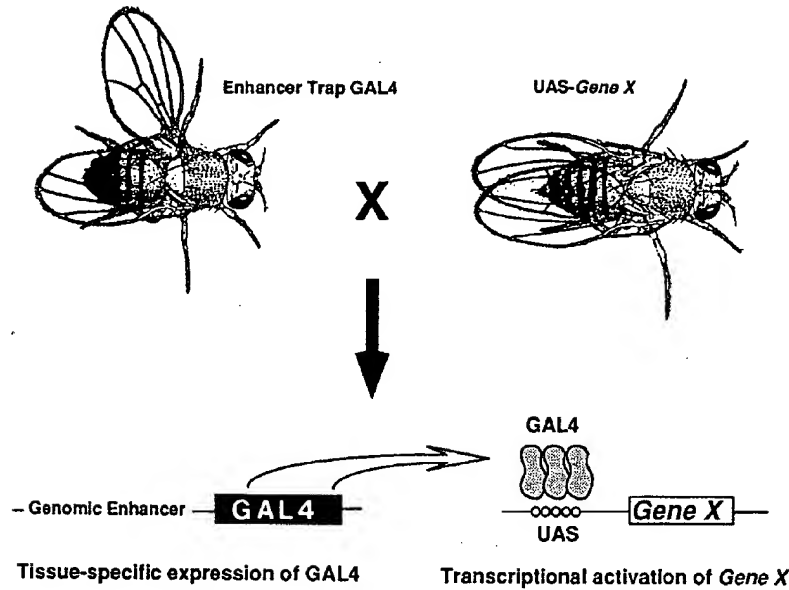


Fig. 1. Directed gene expression in *Drosophila*. To generate transgenic lines expressing GAL4 in numerous cell- and tissue-specific patterns, the GAL4 gene is inserted randomly into the genome, driving GAL4 expression from numerous different genomic enhancers. A GAL4-dependent target gene can then be constructed by subcloning any sequence behind GAL4 binding sites. The target gene is silent in the absence of GAL4. To activate the target gene in a cell- or tissue-specific pattern, flies carrying the target (*UAS-Gene X*) are crossed to flies expressing GAL4 (Enhancer Trap GAL4). In the progeny of this cross, it is possible to activate *UAS-Gene X* in cells where GAL4 is expressed and to observe the effect of this directed misexpression on development.

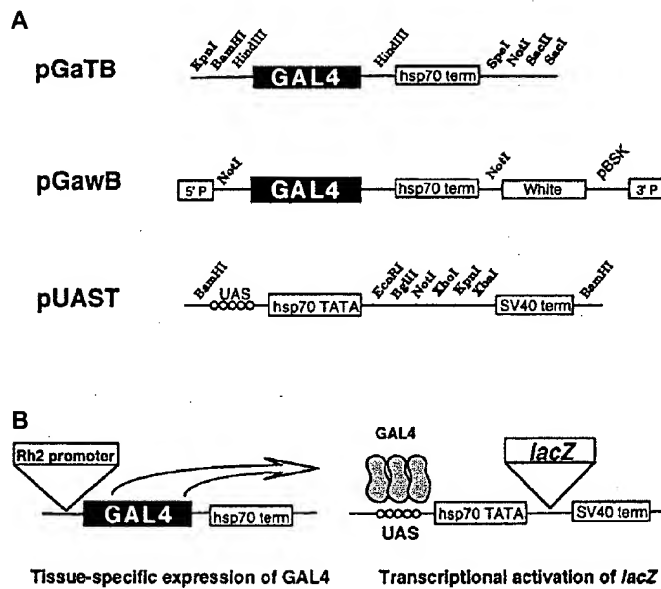
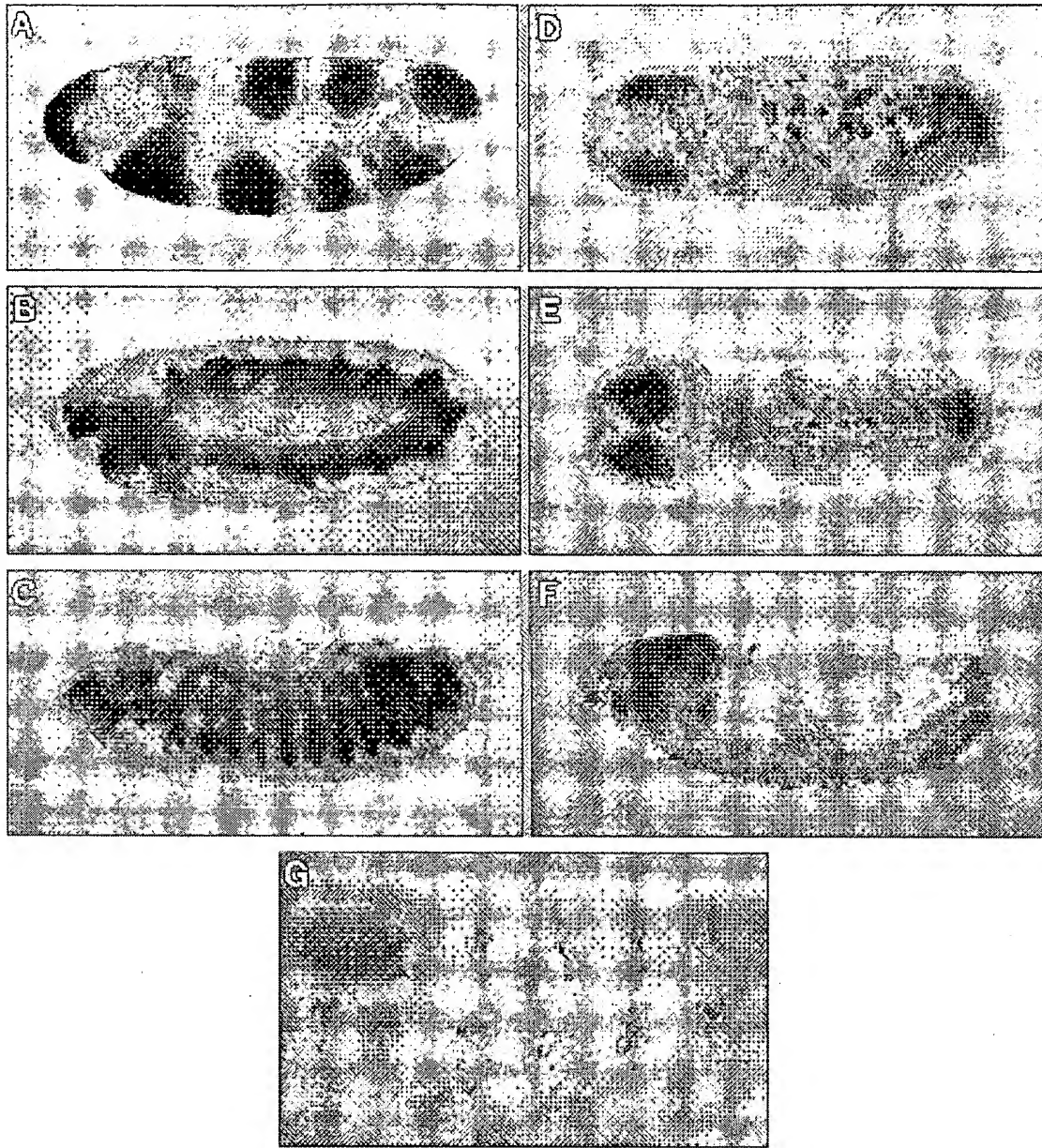


Fig. 2. (A) Vectors for directed gene expression. The vectors pGaTB, pGawB and pUAST are illustrated. To target GAL4 expression to specific cells, promoters can be subcloned upstream of GAL4 at the unique *Bam*HI site of pGaTB. pGawB is an enhancer detection vector that directs expression of GAL4 in a genomic integration site-dependent fashion. pUAST is designed to direct GAL4-dependent transcription of a gene of choice. The sequence is subcloned into a polylinker situated downstream of five tandemly arrayed, optimized GAL4 binding sites, and upstream of the SV40 small t intron and polyadenylation site. Unique restriction sites are indicated in bold letters. (B) GAL4-dependent expression of  $\beta$ -galactosidase in the ocelli. To direct expression of GAL4 to the photoreceptor cells of the ocelli, the promoter of the *Rh2* gene was subcloned upstream of GAL4 in pGaTB. Flies heterozygous for this gene construct (*Rh2-7-2*) were crossed to a line homozygous for the *UAS-lacZ* gene. In the progeny of this cross approx. 50% of the flies express  $\beta$ -galactosidase in the ocelli, as assayed by staining with X-Gal. On the left is the head of a fly that carries both the *Rh2-GAL4* fusion gene and the *UAS-lacZ* reporter construct, on the right is the head of a fly that carries only the *UAS-lacZ* construct.



**Fig. 3.** GAL4 expression patterns generated by enhancer detection. Three enhancer detection/GAL4 lines were crossed to a *UAS-lacZ* line ( $\beta$ g4-1-2) and their progeny were assayed for expression of  $\beta$ -galactosidase by staining with anti- $\beta$ -galactosidase antibodies. (A) Line 1J3: pGawB has inserted at the *hairy* locus and activates expression of  $\beta$ -galactosidase in a pair-rule pattern of seven stripes, and in the head of a stage 11 embryo. (B) Line 24B: this GAL4 insertion directs expression of  $\beta$ -galactosidase first in the presumptive mesoderm, as shown in a stage 11 embryo, and then (C) in the muscle cells of a stage 13 embryo. (D) Line 31-1: this pGawB insertion directs expression of  $\beta$ -galactosidase in neuroblasts at stage 11 (D; dorsal view) and stage 12 (E; dorsal view), and then in neurons in both (F) the central and (G) the peripheral nervous system at stage 13. In all photographs anterior is to the left.

In one line the target gene remains silent in the absence of its activator, in the second line the activator protein is present but has no target gene to activate. This ensures that the parental lines are viable. Only when the two lines are crossed is the target gene turned on in the progeny, and the phenotypic consequences of misexpression (including

lethality) can be conveniently studied. Finally, the method is designed to generate lines that express a transcriptional activator, rather than an individual target gene, in numerous patterns. The target can then be activated in different cell- and tissue-types merely by crossing a single line carrying the desired target to a library of activator-expressing

lines. Thus, a library of different activator-expressing lines can direct each new target gene to be expressed in numerous distinct patterns.

A first step is to generate lines expressing a transcriptional activator in a variety of patterns. It is necessary to use a transcriptional activator that has no endogenous targets in *Drosophila*, so as to express ectopically only the gene of interest. An activator from yeast, GAL4, can activate transcription in flies but only from promoters that bear GAL4 binding sites (Fischer et al., 1988). The GAL4 protein is a potent transcriptional activator in yeast, and the protein has been extensively characterized with respect to both DNA binding and transcriptional activation (reviewed by Ptashne, 1988). The GAL4 binding site has been mutagenized, generating an optimized site to which GAL4 binds with high affinity (Webster et al., 1988). Numerous mutations have been made in the GAL4 transcriptional activation domain, producing activators of varying strengths (Ma and Ptashne, 1987a,b; Gill and Ptashne, 1987; Johnston and Dover, 1988). GAL4 has been shown to activate transcription, not only in *Drosophila*, but also in plants and in mammalian cells, again only from promoters that bear GAL4 binding sites (Fischer et al., 1988; Ma et al., 1988; Kikidani et al., 1988; Webster et al., 1988; Ornitz et al., 1991).

We have taken two approaches to generate different patterns of GAL4 expression. First, GAL4 transcription can be driven by characterized *Drosophila* promoters. The second approach is based on the 'enhancer detection' technique, which was developed as a means of identifying transcriptional regulatory elements in situ in the *Drosophila* genome (O'Kane and Gehring, 1987; Bier et al., 1989; Bellen et al., 1989; Wilson et al., 1989). O'Kane and Gehring (1987) fused the *E. coli lacZ* gene to the weak P-transposase promoter, and showed that this reporter gene could respond to neighboring transcriptional regulatory elements. By fusing the GAL4 coding sequence to the P-transposase promoter, we constructed a vector that, depending upon its genomic site of integration, can direct expression of GAL4 in a wide range of patterns in embryos, larvae and adults. This eliminates the need to link numerous different promoters to the GAL4 gene, and allows expression in novel patterns from enhancers that have not yet been described. In addition, the enhancer detection/GAL4 vector can be mobilized to new genomic sites simply by P-transposition (Cooley et al., 1988; Robertson et al., 1988). In this way, a single transformant can be used to generate a large number of transgenics, each exhibiting a different GAL4 expression pattern.

To create GAL4-responsive target genes, we designed a vector into which genes can be subcloned behind a tandem array of five optimized GAL4 binding sites (hereafter referred to as the UAS, for Upstream Activation Sequences), and upstream of the SV40 transcriptional terminator. It is possible, then: (1) to subclone any sequence behind GAL4 binding sites; (2) to activate that target gene only within cells where GAL4 is expressed and (3) to observe the effect of this aberrant expression on development.

Using the GAL4 system we have been able to generate specific embryonic phenotypes by misexpression of the homeobox-containing transcription factor *even-skipped*. *even-skipped* is involved in determining cell fate, first

during embryonic segmentation, and later during the development of the central nervous system (CNS; Frasch et al., 1987, 1988; Doe et al., 1988). By restricting misexpression within the ectoderm, we have been able to alter epidermal cell fates specifically without affecting central nervous system development. This now permits the separate functions of *even-skipped* in different tissues to be studied independently of one another.

Dominant phenotypes can be generated in adults using the GAL4 system to restrict the expression of potentially lethal products, such as constitutively active proteins. Suppression or enhancement of these phenotypes can then be used to identify interacting gene products, or to establish epistatic relationships. Dominant mutations recovered by classical genetic techniques have proved invaluable in identifying and ordering the components of several signal transduction pathways in *C. elegans* and *Drosophila*. For example, the Ras proteins have been shown to act in determining cell fate in the nematode vulva and in the *Drosophila* compound eye (Han and Sternberg, 1990; Simon et al., 1991). Of the three Ras homologues isolated from flies (Neuman-Silberberg et al., 1984; Brock, 1987), *Dras1*, which is most similar to Ha-ras, appears to act downstream of the receptor tyrosine kinases *sevenless* and *DER* (the *Drosophila* EGF receptor) to determine cell fate during eye development (Simon et al., 1991). *Dras3* is also required in the eye and encodes a homologue of the human *rap1* gene, a possible Ras antagonist (Hariharan et al., 1991). *Dras2* is most similar to R-ras (Lowe et al., 1987), but its role in development has not yet been determined. We have generated dominant, visible phenotypes in adults by targeted expression of constitutively active *Dras2* (Bishop and Corcoran, 1988). Genetic suppression of these phenotypes can now be used to identify mutations in interacting gene products, as a means to elucidate the *Dras2* signal transduction pathway.

## MATERIALS AND METHODS

### GAL4 expression vectors

1. *pGATB* and *pGATN*: we have constructed vectors in which either a unique *NotI* or *BamHI* site is inserted upstream of the GAL4 coding region. The GAL4 coding sequence was excised from vector pLKC15 (a gift from L. Keegan) as a *HindIII* fragment. This fragment extends from a synthetic *HindIII* site inserted approximately 15 nucleotides upstream of the initiator methionine, and includes the complete GAL4 coding sequence and its transcriptional terminator (L. Keegan, personal communication). The *HindIII* fragment was subcloned in the vector pHSREM (Knipple and Marsella-Herrick, 1988) to give plasmid pF18-13, and the heat shock consensus sequences were removed from the promoter by digestion with *BglII* and *EspI*. A unique *BamHI* site was inserted in place of the heat shock boxes to create pGATB. To construct pGATN, a *NotI* site was inserted in place of the heat shock elements. Promoters can now be subcloned upstream of GAL4, and the fusion gene moved from the pHSREM vector backbone into a P-element vector.

2. *Rh2-GAL4*: the *Rh2* promoter (Mismer et al., 1988) was excised from vector pHSS7 as a *BamHI* fragment and subcloned into the *BamHI* site of vector pGATB, creating p25/18XI. The *Rh2-GAL4* fusion gene was then subcloned, as a *KpnI-NotI* fragment, into pCaSpeR4 (a gift from C. Thummel and V. Pirrotta) to make pF72.

3. pGawB: as a first step in creating an enhancer detection GAL4 vector, we modified the enhancer detection *lacZ* vector, plwB (Wilson et al., 1989). To remove the *NotI* site in the vector, plwB was digested with *NotI* and the 5' overhanging ends were filled using T4 polymerase. The resultant blunt ends were then ligated to make plasmid p41-4. To remove the P-transposase-*lacZ* fusion gene, p41-4 was digested with *HindIII*, the *HindIII* fragment was removed and the plasmid was religated, forming p41-4-H3-1. A short linker oligonucleotide, formed by annealing the sequences: 5'-AGCTTGGTTAACGCGGCCGC-3' and 3'-ACCAATTGCGCGCGCTCGA-5', was then subcloned into the *HindIII* site of p41-4-H3-1. In the resultant plasmid, p41-4Hpa, the *HindIII* site is maintained and a unique *HpaI* site is introduced.

To reconstitute the 5' end of the P-element and the P-transposase promoter, we synthesized an oligonucleotide that extends from the *HindIII* site in the 5' end of the P-element to nucleotide 140, followed by the sequence CGGCCGC, to create a *NotI* site. The oligonucleotide was subcloned as a *HindIII*-blunt ended fragment into p41-4Hpa cut with *HindIII* and *HpaI*, to create p41-4Hpa14.

As a final step, the GAL4 coding sequence followed by the *hsp70* terminator was isolated from pGaTN by digestion with *NotI*. The *NotI* fragment was subcloned into the unique *NotI* site of p41-4Hpa14 to create pGawB.

#### GAL UAS fusion genes

1. pUAST: we constructed a vector into which genes can be subcloned behind the GAL UAS. A fragment containing five optimized GAL4 binding sites (the 'Scal' site' 17-mer; Webster et al., 1988) and a synthetic TATA box (Lillie and Green, 1989) separated by a unique *NotI* site from the SV40 terminator was excised from pF40X2-1 by digestion with *NsiI* and *SpeI*. The fragment was subcloned into the P-element vector pCaSpeR3 (a gift from C. Thummel and V. Pirrotta), cut with *PstI* and *XbaI*, to give plasmid pF91. This vector was used to drive expression of *Dras2<sup>Val14</sup>*, but appeared to be inefficient in promoting transcription, and so was modified as follows.

pF91 was digested with *XbaI*, and a fragment spanning the TATA box was removed. This was replaced by a fragment containing the *hsp70* TATA box, generated as a PCR product using pCaSpeR-hs (a gift from C. Thummel and V. Pirrotta) as a template. The PCR product begins with an *NheI* restriction site, extends from eleven nucleotides upstream of the *hsp70* TATA box to the *SacII* site in pCaSpeR3, and is followed by restriction sites for *XhoI*, *KpnI* and *XbaI*. The resulting vector, named pUAST, consists of five tandemly arrayed, optimized GAL4 binding sites, followed by the *hsp70* TATA box and transcriptional start, a polylinker with unique restriction sites for *EcoRI*, *BglII*, *NotI*, *XhoI*, *KpnI* and *XbaI*, and the SV40 small t intron and polyadenylation site.

2. *UAS-lacZ*: an *Adh-lacZ* fusion gene was removed from pCaSpeR-AUG- $\beta$ -gal (Thummel et al., 1988) by digestion with *EcoRI* and *XbaI*, and was subcloned in pUAST.

3. *UAS-even-skipped*: the *even-skipped* gene was subcloned as a *HincII* fragment from plasmid pEAI (a gift from R. Warrior and M. Levine) into the plasmid pF91 that had been cut with *NotI* and made blunt using T4 polymerase, to give plasmid pF100. The *HincII* fragment contains the 5' end of the *even-skipped* cDNA (from an *XhoI* site to a *PstI* site) fused to the 3' end of the genomic *even-skipped* sequence (from the *PstI* site to an *EcoRI* site; MacDonald et al., 1986; Frasch et al., 1987). The *even-skipped* gene was then excised from pF100 as a *SmaI-XbaI* fragment and subcloned into pUAST that had been digested with *NotI* and *XbaI*, and the *NotI* site made blunt with T4 polymerase.

4. *UAS-Dras2<sup>Val14</sup>*: the *Dras2<sup>Val14</sup>* gene was excised from plasmid pIB120 7.7 *XhoI*#6 (a gift from J. Bishop and V. Corces) as a *HpaI-XhoI* fragment and was subcloned into plasmid pF91 that

had been digested with *KpnI* and *XhoI*, and the *KpnI* site made blunt with T4 polymerase.

#### Injections

Transgenic lines were generated by injection of CsCl banded DNA, at a concentration of 600  $\mu$ g/ml, into embryos of strain y w; +/+; *Sb*, P[ry+,  $\Delta$ 2-3]/TM6, *Ubx* (Robertson et al., 1988) using standard procedures (Santamaria, 1986; Spradling, 1986).

#### Enhancer detection screen

Flies were raised on standard *Drosophila* medium at 25°C. Descriptions of balancers and mutations that are not described in the text can be found in Lindsley and Zimm (1992).

An enhancer detection screen was carried out to recover lines that express GAL4 in a cell- or tissue-specific manner. Insertion lines were generated by mobilizing a single X-linked enhancer detection GAL4 vector, pGawB. The first insertion line we used is hemizygous lethal and is carried over an *FM7* balancer chromosome (*GAL4-lethal/FM7*). We also mobilized a pGawB insertion from the  $\Delta$  2.3, *Sb* chromosome. The P-element transposons were mobilized using the 'jumpstarter' strain P[ry+;  $\Delta$ 2-3], which carries a defective P-element on the third chromosome at 99B (Laski et al., 1986; Robertson et al., 1988; Cooley et al., 1988). This P-element expresses high levels of a constitutively active transposase, but cannot itself transpose. The frequency with which new pGawB insertion lines were recovered was much lower than that previously reported for a similarly sized enhancer detection/*lacZ* P-element (N. P., unpublished data). This might be attributed to the alterations made in the sequence of the 5' end of the P-element pGawB that allow GAL4 to be expressed from its own AUG, rather than as a P-transposase-GAL4 fusion protein.

Insertions segregating with the X chromosome were detected by examining the segregation of w<sup>+</sup>. Autosomal insertions were mapped by standard genetic methods using the two stocks: w; +/+; TM3, *Sb/CxD* and w; *CyO/Sco*; +/+.

Each of the 220 lines was crossed to a line carrying either the 17-*hsp70/lacZ* (Fischer et al., 1988) or the *UAS-lacZ* reporter gene. Embryos from the cross were collected on agar/molasses plates and stained for  $\beta$ -galactosidase expression with anti- $\beta$ -galactosidase antibodies. A subset of the lines were also examined for  $\beta$ -galactosidase expression in imaginal discs by staining with X-Gal.

#### Antibody immunocytochemistry and X-Gal staining

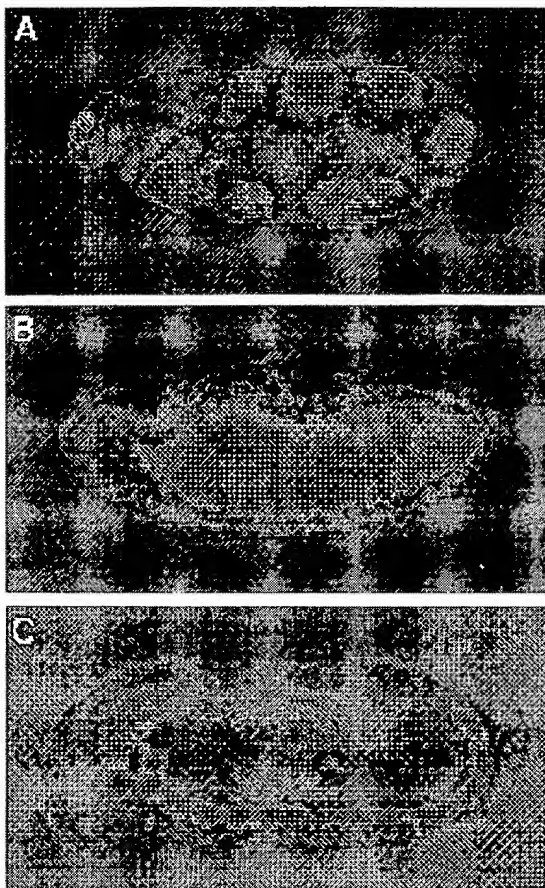
Embryos were dechorionated in 50% Clorox, fixed for 10 minutes in a 1:1 mix of heptane and 3.7% formaldehyde in PBS, 0.1% Triton X-100, and the vitelline membranes were removed by shaking in heptane/methanol. The embryos were incubated overnight at 4°C in a 1:1000 dilution of rabbit anti- $\beta$ -galactosidase antibody (a gift from B. Holmgren), or a 1:1 dilution of mouse anti-*even-skipped* antibody (a gift from N. Patel). After washing in PBS, 0.1% Triton X-100, embryos were incubated for 2 hours in biotinylated horse anti-mouse antibody (Vector Laboratories) at a 1:500 dilution, and then for 1 hour in avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories). Peroxidase was detected using diaminobenzidine (0.3 mg/ml in PBS, 0.1% Triton X-100) as a substrate. Embryos were then dehydrated in ethanol and mounted in methyl salicylate.

Imaginal discs were dissected in PBS, 0.1% Triton X-100 and fixed in 1% glutaraldehyde for 15 minutes. They were then stained in a solution of 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> [pH 7.2], 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 3 mM K<sub>4</sub>[FeII(CN)<sub>6</sub>], 3 mM K<sub>3</sub>[FeIII(CN)<sub>6</sub>] containing a 1/50 dilution of X-Gal (25 mg/ml in dimethyl formamide). After washing in PBS, 0.1% Triton X-100, discs were mounted in 70% glycerol.

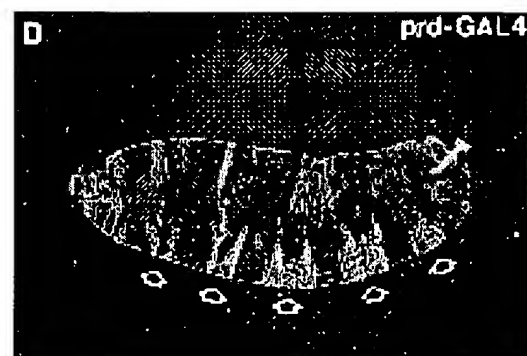
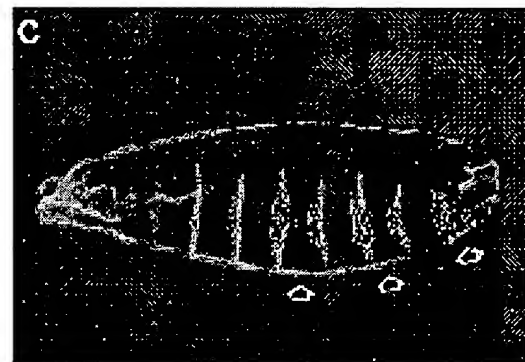
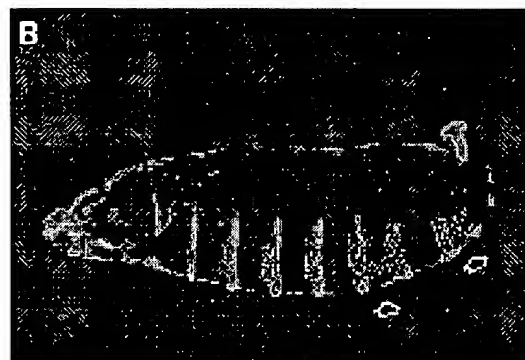
#### In situ hybridization

Single stranded DNA probes were prepared as described by Patel





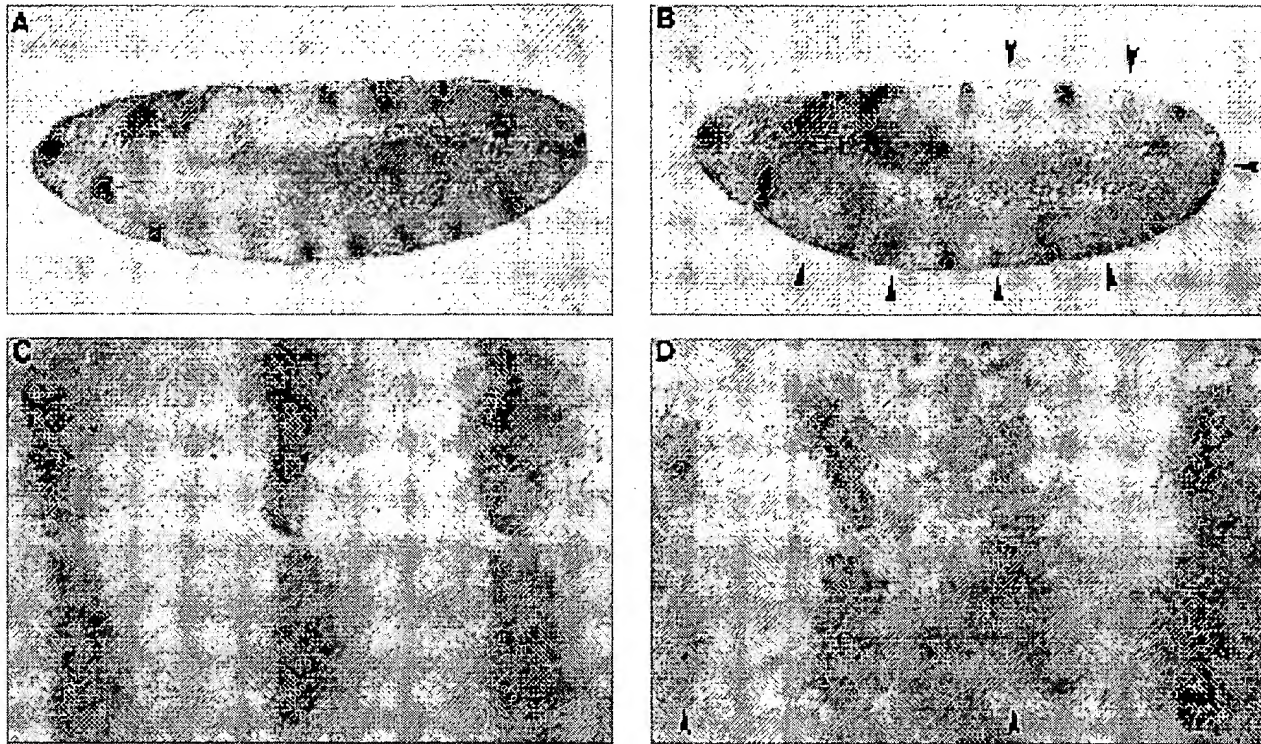
**Fig. 4.** GAL4-dependent misexpression of *even-skipped*. We used the same three GAL4-expressing lines shown in Fig. 3 to direct ectopic expression of the *even-skipped* gene. Lines 1J3, 24B and 31-1 were crossed to a *UAS-even-skipped* line (*eve-2-7-1*) and the progeny of the cross were stained with anti-*even-skipped* antibodies (3C10; N. Patel and C.S. Goodman, unpublished data). (A) Line 1J3 directs *even-skipped* expression in seven stripes and in the head of a stage 9 embryo (compare the ectopic *even-skipped* expression shown here with the  $\beta$ -galactosidase expression in Fig. 3A). (B) Line 24B drives *even-skipped* expression in the muscles of a stage 13 embryo (compare with the  $\beta$ -galactosidase expression pattern in Fig. 3C). (C) Line 31-1 activates *UAS-even-skipped* in cells throughout the central nervous system of a stage 12 embryo (see Fig. 3F for the comparable  $\beta$ -galactosidase expression pattern). In all photographs anterior is to the left, ventral at the bottom.



and Goodman (1992). In situ hybridization to whole-mount embryos was carried out as described by Ephrussi et al. (1991), except that embryos were fixed in 3.7% formaldehyde, without DMSO. In addition, the 1 hour incubation in methanol/DMSO at  $-20^{\circ}\text{C}$  was omitted. Embryos were cleared in 50% glycerol for several hours and then mounted in 70% glycerol.

#### Cuticle preparations

Cuticles were prepared and mounted as described by Struhl (1989).



**Fig. 6.** Misexpression of *even-skipped* selectively represses *wingless* expression. The loss of *wingless* between 6 and 9.5 hours of development leads to the appearance of extra denticles (Bejsovec and Martinez Arias, 1991; Dougan and DiNardo, 1992). Since misexpression of *even-skipped* gives a similar phenotype, we assayed whether ectopic *even-skipped* represses *wingless* transcription. *wingless* transcription in (A) a wild-type embryo at stage 11 (5:20–7:20 hours AEL), as assayed by in situ hybridization, and in B an embryo in which *UAS-even-skipped* expression is driven by *paired-GAL4* (stage 10; 4:20–5:20 hours AEL). In B *wingless* transcription is repressed in alternating segments throughout the embryo (arrowheads). (C) A wild-type embryo (about stage 11) stained with anti-*wingless* and anti-*even-skipped* antibodies. At this stage of development *wingless*, but not *even-skipped*, is expressed in stripes. (D) An embryo (about stage 11) in which *UAS-even-skipped* expression is driven by *hairy-GAL4*, stained with anti-*wingless* and anti-*even-skipped* antibodies. The *wingless* expressing cells are brown and the *even-skipped* expressing cells are a very pale purple. *wingless* is repressed (arrowhead) in those cells that ectopically express *even-skipped*. The anti-*even-skipped* staining is underdeveloped so as not to obscure *wingless* staining. In all photographs, anterior is to the left; A and B are lateral views; C and D are dorsal views of germ band extended embryos.

**Fig. 5.** Ectopic expression of *even-skipped* leads to segmental fusions. The GAL4-expressing line 1J3 directs expression of *even-skipped* in a pattern corresponding to that of the *hairy* gene (Fig. 4A). Cuticles prepared from these embryos exhibit fusions in alternating pairs of segments. (A) An embryo in which abdominal segments seven and eight are fused (arrow). (B) An embryo in which abdominal segments five and six, then seven and eight are fused (arrows), and (C) an embryo in which abdominal segments three and four, five and six, and seven and eight are fused (arrows). These phenotypes were observed in 68 out of 103 embryos in one experiment. More extensive fusions (e.g. A3–A4 and A5–A8; A6–A8; A3–A8) were observed in 15 embryos, while the remainder appeared wild-type. To show that the varied expressivity is not a general property of GAL4-mediated activation, but is due rather to GAL4 expression from the *hairy* promoter, we used a *paired-GAL4* construct to drive expression of *UAS-even-skipped*. In the progeny of this cross, as shown in D, 75% of the embryos show alternating segmental fusions along the entire length of the cuticle (arrows indicate thoracic segments T2–3 and abdominal segments A1–A2, A3–A4, A5–A6 and A7–A8). In all photographs anterior is to the left.

## RESULTS

### GAL4 expressed from the *Rh2* promoter activates transcription in the photoreceptor cells of the ocelli

We constructed a vector into which promoters can be inserted upstream of the GAL4 coding sequence (pGaTB; Fig. 2A). Transcriptional regulatory sequences are subcloned into the unique *Bam*HI site to create a gene that includes the promoter of choice fused to the GAL4 coding sequence, which is in turn linked to the transcriptional termination sequence of the *hsp70* gene.

To test whether GAL4-activated transcription is both specific and efficient, we used the promoter of the *Rh2* gene to drive GAL4 expression. The *Rh2* promoter is active in the photoreceptor cells of the ocelli, which are three light sensing organs located on the top of the *Drosophila* head (Mismar et al., 1988; Pollock and Benzer, 1988). Transgenic lines carrying the *Rh2-GAL4* gene were crossed to a line that carries the *E. coli lacZ* gene regulated by GAL4 binding sites (*UAS-lacZ*; see below; Fig. 2B). In the progeny of this cross GAL4 should be expressed in the pho-



toreceptor cells of the ocelli, where it should activate transcription of the *UAS-lacZ* gene.

Fig. 2B shows the heads of two flies: on the left is a fly that carries both the *Rh2-GAL4* and the *UAS-lacZ* fusion genes and on the right, one that carries only the *UAS-lacZ* gene. In an assay for  $\beta$ -galactosidase expression, the ocelli of flies carrying both the *Rh2-GAL4* and the *UAS-lacZ* fusion genes stain dark blue after a 15 minute incubation with the chromogenic substrate X-Gal, whereas the ocelli of flies that carry only the *UAS-lacZ* gene do not stain.

### Enhancer detection to express GAL4 in cell- and tissue-specific patterns

To generate many patterns of GAL4 expression rapidly, without having to rely on the availability of suitable promoters, we constructed an enhancer detection vector in which GAL4 transcription is directed by the weak promoter of the P-transposase gene (pGawB; Fig. 2A). With this vector, the enhancerless GAL4 gene can be integrated at random in the genome, bringing it under the control of a diverse array of genomic enhancers. The previously described enhancer detection vectors express a P-transposase/ $\beta$ -galactosidase fusion protein that is directed to the nucleus by a nuclear localization signal within the P-transposase sequence (O'Kane and Gehring, 1987; Bier et al., 1989; Bellen et al., 1989; Wilson et al., 1989). Since the amino terminus of GAL4 directs both nuclear localization and DNA binding (Silver et al., 1984; Keegan et al., 1986), the enhancer trap/GAL4 vector, pGawB, was designed to express GAL4 from its own AUG, but is otherwise similar to the *lacZ* enhancer detection vector, plwB (Wilson et al., 1989; see Fig. 2A and Materials and methods).

To make target genes responsive to transcriptional activation by GAL4, we constructed a vector into which genes can be subcloned downstream of five tandemly arrayed, high affinity GAL4 binding sites and upstream of the SV40 transcriptional terminator (pUAST; Fig. 2A). To test pUAST, the *lacZ* gene was inserted within the polylinker sequence to create the GAL4-responsive gene, *UAS-lacZ* (Fig. 2B).

Transgenic lines carrying pGawB were generated by P-element transformation. To ensure that these transformants express GAL4, each line was crossed to flies carrying *UAS-lacZ*, and the resultant progeny were stained for  $\beta$ -galactosidase activity. A line with pGawB inserted within an essential gene on the X-chromosome (*GAL4-lethal*) was used as a starter line to mobilize pGawB to different sites in the genome by introduction of a constitutively active P-transposase gene ( $\Delta 2-3$ ; Laski et al., 1986; Robertson et al., 1988; Cooley et al., 1988; see Materials and methods).

We examined 220 independent insertion lines. To identify the cells in which GAL4 activates transcription, each insertion line was crossed to the *UAS-lacZ* line, and the progeny were stained with anti- $\beta$ -galactosidase antibodies. In this screen, 61% of the GAL4 insertions activate *UAS-lacZ* in a specific embryonic pattern. The distribution of patterns is shown in Table 1. In addition to activating expression in specific embryonic patterns, 51% of the insertions also activate transcription in the salivary glands and 28% activate in the salivary glands alone, suggesting that in constructing the GAL4 vector we may have generated a

Table 1. Patterns of GAL4-directed expression in embryos

No. of strains	No staining	Salivary glands	Other tissues	%
23	+	-	-	10.45
62	-	+	-	28.2
112	-	+	+	50.9
23	-	-	+	10.45
Total	220			100.00

position-dependent salivary gland enhancer. The earliest time in embryonic development that we have been able to detect GAL4-mediated expression is just after gastrulation, between 3.5 and 4 hours after fertilization.

### GAL4 expression patterns

Many of the GAL4 lines we generated can be used to activate genes in specific embryonic tissues. In Fig. 3 we show a representative group of GAL4-expressing lines. The GAL4 insertion 1J3 activates transcription of *UAS-lacZ* in a pair-rule pattern of seven stripes, as assayed by staining with anti- $\beta$ -galactosidase antibodies (Fig. 3A). This expression pattern resembles that of the gene *hairy* with respect to both the number and placement of the stripes, and the pattern of expression in the head (Carroll et al., 1988; Hooper et al., 1989). In common with the *hairy* protein expression pattern, stripe four is fused to stripe three at gastrulation and is somewhat weaker than the other stripes. Ectodermal *hairy* expression begins to decay at gastrulation, while expression endures within the mesoderm until stage 9/10, at the end of germ band extension (Hooper et al., 1989). Ectodermal  $\beta$ -galactosidase expression directed by GAL4 insertion 1J3 persists through germ band extension, and eventually fades. Expression is maintained in the mesoderm and is later observed within muscle cells. A *lacZ* enhancer detection vector inserted at the *hairy* locus gives a  $\beta$ -galactosidase expression pattern similar to that directed by line 1J3. The prolonged period of expression in stripes might be due to increased mRNA stability, or to perdurance of  $\beta$ -galactosidase in the former case, and of the GAL4 protein and the target gene product in the latter.

When the 1J3 insertion is made homozygous, viable adults exhibiting extra scutellar bristles are generated, a phenotype associated with several *hairy* alleles (Ingham et al., 1985). Adults that carry the 1J3 insertion in *trans* to *hairy* allele *h<sup>5HO7</sup>* also exhibit extra scutellar bristles, suggesting that in line 1J3 pGawB has inserted at the *hairy* locus.

Expression of GAL4 by insertion 24B causes *UAS-lacZ* to be transcribed first in the presumptive mesoderm, as observed at germ band extension (Fig. 3B), and then in muscle cells (Fig. 3C). Insertion 31-1 activates *UAS-lacZ* in neuronal precursor cells, or neuroblasts, at stage 11 (Fig. 3D,E) and then within neurons of both the central and peripheral nervous systems (Fig. 3F,G). Other embryonic tissues or cells in which GAL4 can activate *UAS-lacZ* include the amnioserosa, the foregut, midgut and hindgut, the pharynx, the malpighian tubules, vitellophages, oenocytes and glia.

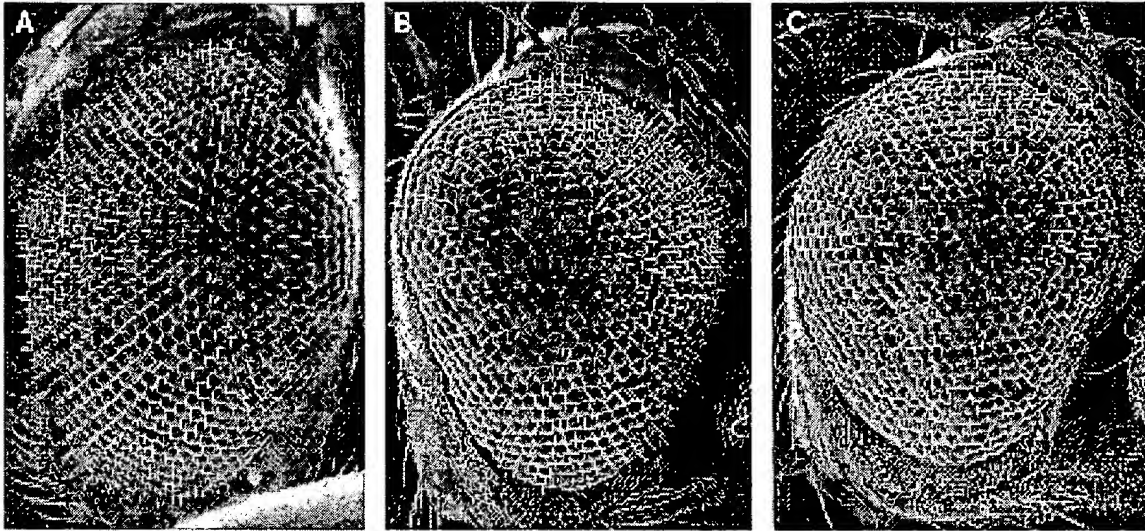


Fig. 7. GAL4-dependent expression of *Dras2<sup>Val14</sup>* leads to a rough eye phenotype. Scanning electron micrographs of compound eyes from flies with the following genotypes: (A) wild type; (B) GAL4 insertion 32B driving expression of *UAS-Dras2<sup>Val14</sup>*, and (C) GAL4 insertion 69B driving expression of *UAS-Dras2<sup>Val14</sup>*, demonstrating the roughening caused by targeted expression of *Dras2<sup>Val14</sup>*.

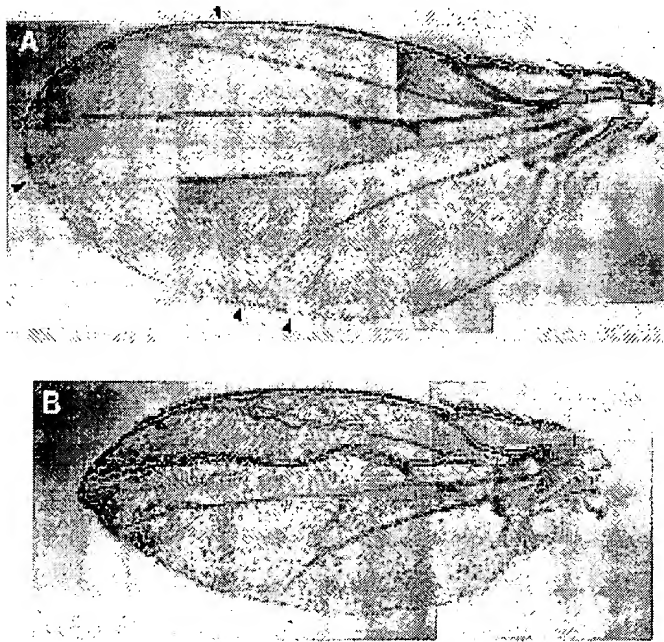


Fig. 8. Targeted expression of *Dras2<sup>Val14</sup>* disrupts wing development. (A) A wing from a fly in which GAL4 insertion 32B drives *UAS-Dras2<sup>Val14</sup>* expression: the longitudinal veins bifurcate at the wing margin (arrow), and extra vein material is evident (arrow). (B) A more severe phenotype is seen in flies where GAL4-insertion 69B drives *UAS-Dras2<sup>Val14</sup>* expression: the wing is reduced in size and exhibits broadened and disorganized veins. These flies rarely eclose.

### Altering cell fates by targeted expression of *even-skipped*

Targeted misexpression is a promising approach for examining the roles of different regulatory proteins in specifying cell fates. We used GAL4 to target expression of the *even-skipped* gene in an attempt to direct specific cell fate changes. *even-skipped* encodes a homeodomain protein that is involved in determining cell fate in the ectoderm, during embryonic segmentation, and later in the central nervous system (Frasch et al., 1988; Doe et al., 1988). *even-skipped* is expressed at the blastoderm stage in seven stripes, cor-

responding to the odd-numbered parasegments. By stage 7, at 3 hours of development, the stripes have faded and expression is not observed again until after germ band retraction when *even-skipped* can be detected in the central nervous system in about fourteen neurons per hemisegment (Patel et al., 1989).

To test if GAL4 can activate *UAS-even-skipped* to levels that result in embryonic phenotypes, transgenic lines carrying *UAS-even-skipped* were crossed to the GAL4-expressing lines shown in Fig. 3. The progeny of each cross were then stained with anti-*even-skipped* antibodies (Fig. 4). Fig. 4A shows GAL4-insertion 1J3 activating *even-*

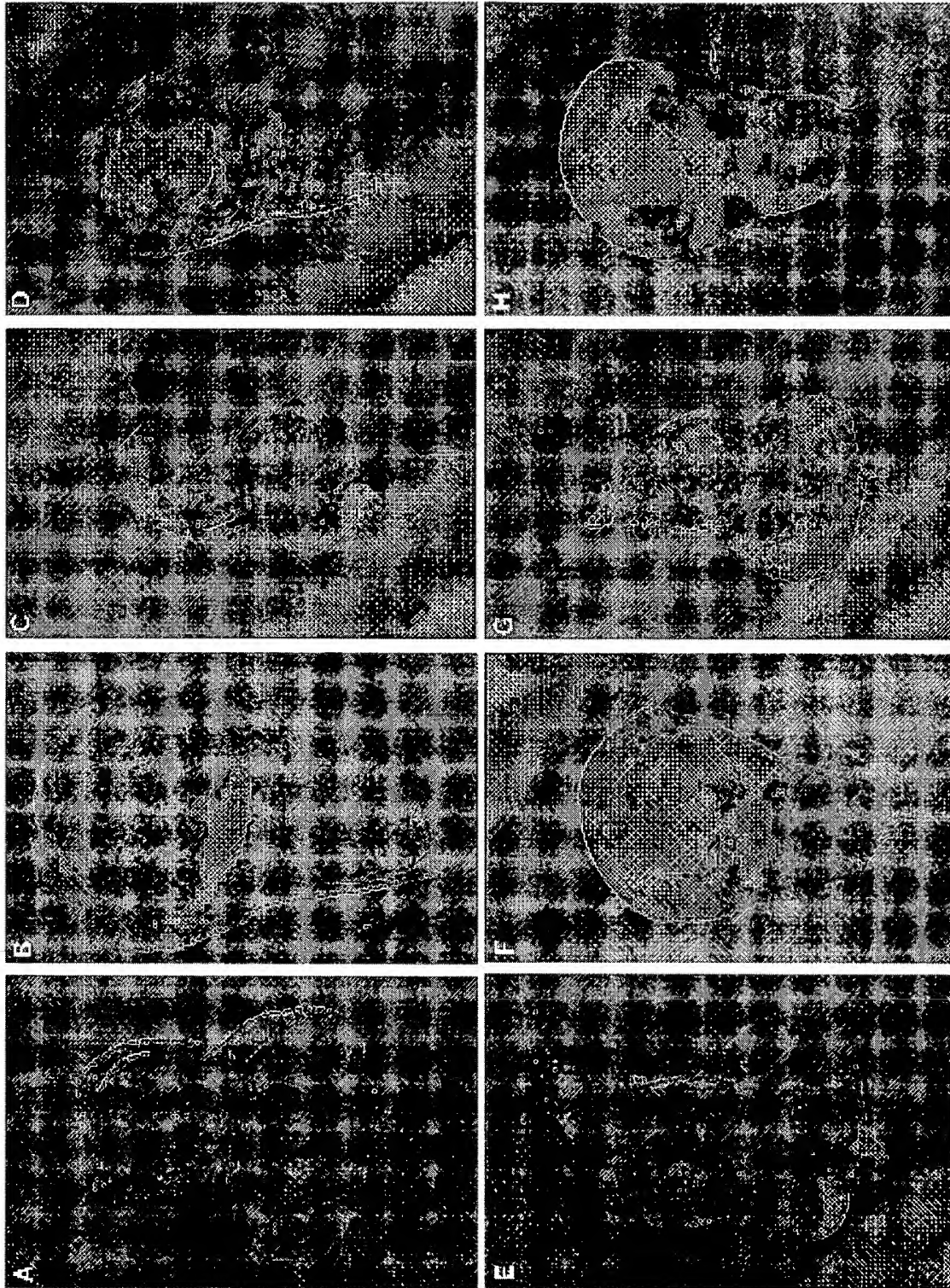


Fig. 9. GAL4 expression in imaginal discs. Four GAL4-expressing lines that give visible phenotypes when driving expression of *UAS-Dras2<sup>Val14</sup>* were crossed to a *UAS-lacZ* line ( $\beta$ gal-1-2), and the progeny were allowed to develop to the third instar larval stage. The larvae were then dissected and their imaginal discs were stained with X-GAL to detect  $\beta$ -galactosidase activity. Shown here are the eye and wing imaginal discs from: (A, B) line 71B, which gives rise to a *UAS-Dras2<sup>Val14</sup>*-dependent wing phenotype; (C, D) line 30A, which gives a *UAS-Dras2<sup>Val14</sup>*-dependent wing phenotype; (E, F) line 32B, which gives rise to a *UAS-Dras2<sup>Val14</sup>*-dependent phenotype in both the eye and the wing (see Figs 7B, 8A); (G, H) line 69B, which also gives a *UAS-Dras2<sup>Val14</sup>*-dependent eye and wing phenotype (see Figs 7C, 8B). In all photographs anterior is up.

*skipped* in the pair rule stripes corresponding to the *hairy* expression pattern. By this stage of development (stage 9, 3:40-4:20 hours) *even-skipped* expression would normally have faded, but expression persists due to GAL4-directed transcription. Fig. 4B shows GAL4 insertion 24B directing *even-skipped* expression in embryonic muscle. In Fig. 4C, *UAS-even-skipped* is activated by GAL4 insertion 31-1, causing *even-skipped* to be expressed throughout the nervous system, rather than in a specific subset of neurons. In each of these crosses, misexpression of *even-skipped* results in embryonic lethality.

Restricting expression within the animal permits cell fates to be altered in a chosen tissue or cell-type. Using the GAL4-insertion line 1J3, we can misexpress *even-skipped* in the ectoderm in the *hairy* pattern (Fig. 4A). In wild-type embryos at the cellular blastoderm stage, *even-skipped* is expressed in the odd-numbered parasegments, partly overlapping the stripes of *hairy* expression. The anterior border of *hairy* expression lies one to two cells anterior to the border of *even-skipped* expression (Carroll et al., 1988; Hooper et al., 1989) and thus encompasses the *wingless*-expressing cells. Between 6 and 9.5 hours of development, *wingless* is required to instruct cells to secrete naked cuticle: the loss of *wingless* in this time window leads to the appearance of extra denticles (Bejsovec and Martinez Arias, 1991; Dougan and DiNardo, 1992). One function of *even-skipped* during segmentation is to repress *wingless* transcription (reviewed by Ingham and Martinez Arias, 1992). Misexpression of *even-skipped* within the *wingless*-expressing cells might therefore switch off *wingless* transcription and effect a cell fate change such that cells that would normally secrete naked cuticle instead produce denticles.

Using the *hairy-GAL4* insertion line (1J3) we have activated *UAS-even-skipped* so as to expand the stripes of *even-skipped* expression anteriorly, into the *wingless*-expression domain, and to prolong *even-skipped* expression beyond the time when endogenous *even-skipped* has faded. This pattern of expression results in embryonic lethality and cuticles prepared from the embryos exhibit extra denticles between alternating segments, as shown in Fig. 5. Ectopic denticles can be seen in the normally naked region between abdominal segments three and four, five and six, and seven and eight (Fig. 5A-C). The expressivity of this phenotype is variable with respect to the number of segmental fusions observed in each embryo, with fusions occurring primarily in the most posterior segments. We attribute this variability to GAL4 expression directed by the *hairy* promoter, since a *paired-GAL4* construct (L. Fasano and C. Desplan, unpublished) driving expression of *UAS-even-skipped* causes alternating segmental fusions along the entire length of the embryo (Fig. 5D).

By targeting *even-skipped* transcription to the *wingless*-expressing cells in alternating segments we are able to elicit a change in epidermal cell fate within the naked region of the embryo. Since *wingless* is required to instruct cells to secrete naked cuticle, this phenotype suggests that *wingless* is being repressed. We assayed *wingless* expression in embryos in which *even-skipped* is ectopically expressed (Fig. 6). When *UAS-even-skipped* is activated by *paired-GAL4*, *wingless* transcription is turned off in alternating segments along the entire length of the embryo (Fig. 6B).

Activating *UAS-even-skipped* with *hairy-GAL4* leads to the repression of alternate *wingless* stripes primarily in the posterior abdominal region of the embryo (Fig. 6D).

### Generation of dominant adult phenotypes by directed expression of activated *Dras2*

We have used the GAL4 activation system to target expression of a constitutively active form of the *Dras2* protein. Our goal was first to establish viable transformant lines carrying the *UAS-activated Dras2* fusion gene, and second to generate stable *Dras2*-dependent phenotypes to be used in genetic screens. One advantage of a two-part activation system (eg. Fischer et al., 1988; Khillan et al., 1988; Byrne and Ruddle, 1989; Ornitz et al., 1991) is the ability to study the consequences of expressing products that might be toxic to the animal (Kunes and Steller, 1991). Using the GAL4 system, genes encoding lethal products can be subcloned into pUAST, where they are silent until GAL4 is introduced. Lines bearing the *UAS-gene* fusion are thus viable until crossed to a GAL4-expressing line. Only the progeny of such a cross will exhibit phenotypes resulting from the activity of the gene. Next, transcription of the gene can be targeted to non-essential tissues, to permit the generation of viable adults with visible phenotypes.

With the intention of generating a constitutively activated form of *Dras2*, Bishop and Corces (1988) converted the glycine residue at position 14 to valine, to produce *Dras2<sup>Val14</sup>*. This mutation, by analogy with mutations at position 12 of mammalian Ras, is thought to activate the protein. Transcription of *Dras2<sup>Val14</sup>* from a heat-shock promoter during third instar larval development kills 60-80% of the population. Surviving flies show eye and wing defects. Transcription of *Dras2<sup>Val14</sup>* from the *Dras2* promoter results in variable eye and wing defects, suggesting that *Dras2* may normally function in both eye and wing development (Bishop and Corces, 1988). The flies also exhibit reduced viability and fertility. Fortini et al. (1992) expressed *Dras2<sup>Val14</sup>* in the developing eye from the *sevenless* promoter, also producing a rough eye phenotype. In contrast to *Dras1*, *Dras2* does not appear to act downstream of *sevenless*, but appears to function in a separate signal transduction pathway.

We were able to generate several independent transgenic lines carrying the *UAS-Dras2<sup>Val14</sup>* fusion gene. All of these lines are viable and show no visible phenotypes in the absence of GAL4, suggesting that the *UAS-Dras2<sup>Val14</sup>* gene is silent. A *UAS-Dras2<sup>Val14</sup>* transgenic line (R52-a) was then crossed to several GAL4-expressing lines (1J3, 24B, 69B, 32B, 30A and 71B). In two crosses the progeny arrest as pupae (1J3 and 24B; data not shown); in one, as pupae and young adults (69B; Figs 7C, 8B), and in three crosses adult phenotypes are apparent (30A, 71B and 32B; Figs 7B, 8A). This suggests that the phenotypes we observe are due to GAL4 expression in the imaginal discs. To confirm this, we assayed for GAL4 expression in the imaginal discs of four of the GAL4 insertion lines and compared the GAL4 expression patterns with the *Dras2<sup>Val14</sup>*-dependent phenotypes produced. Fig. 9 shows the GAL4-dependent  $\beta$ -galactosidase expression pattern observed in the eye and wing imaginal discs of lines 71B, 30A, 32B and 69B.

In crosses where GAL4-insertions 71B and 30A activate



*UAS-Dras2<sup>Val14</sup>* flies show a mild wing defect, such that the wings appear slightly opaque (data not shown). Both of these lines express GAL4 in the wing imaginal disc (Fig. 9B,D, respectively). No obvious eye defects are observed, as might be expected, given the restricted GAL4 expression pattern in the eye imaginal disc in these lines (Fig. 9A,C, respectively). Activating *UAS-Dras2<sup>Val14</sup>* with GAL4-insertions 32B and 69B results in flies with a more severe wing defect, as well as a rough eye phenotype (Figs 7, 8). The wings are not only opaque, but also exhibit vein defects. Lines 32B and 69B express GAL4 at high levels in both the wing and the eye imaginal discs (for line 32B, compare the eye disc in Fig. 9E with the eye in Fig. 7B, the wing disc in Fig. 9F with the wing in Fig. 8A; for line 69B, compare Figs 9G and 7C for the eye, and Figs 9H and 8B for the wing). The high level of *UAS-Dras2<sup>Val14</sup>* expression driven by GAL4-insertion 69B results most often in pupal lethality, whereas expression driven by 32B leads to viable adults in which the *Dras2<sup>Val14</sup>* phenotype is fully penetrant (in a sample of 100 flies, all showed the *Dras2<sup>Val14</sup>*-dependent eye and wing phenotype).

Using enhancer detection/GAL4 insertion lines we have observed *Dras2<sup>Val14</sup>*-dependent phenotypes in the wing alone, or in both the wing and the eye. We can also generate a rough eye phenotype, in the absence of a wing defect, by activating *UAS-Dras2<sup>Val14</sup>* in the developing eye using GAL4 expressed from the *sevenless* promoter (*sev-<sup>less</sup>-GAL4*, K. Basler, unpublished; A. B. and N. P., unpublished data). Since these phenotypes are stable and reproducible, in contrast to those generated by expression of *Dras2<sup>Val14</sup>* from a heat shock promoter, genetic screens can now be carried out with the aim of identifying second-site mutations that affect other proteins in the *Dras2* signal transduction pathway.

## DISCUSSION

### The GAL4 activation system

We have developed a two-part system for targeting gene expression in *Drosophila* that allows transcription to be restricted to specific cells or tissues in embryos, larvae and adults. Using enhancer detection we have generated transgenic lines that express the yeast transcriptional activator GAL4 in numerous different patterns and at various stages of development. To activate a gene specifically in a particular cell or tissue, we constructed target genes in which transcription is driven by GAL4 binding sites. Transgenic flies carrying the target gene are then crossed to a GAL4-expressing line, producing progeny in which the target gene is transcribed in a specific GAL4-dependent pattern.

The GAL4 system can be used to express any gene of interest ectopically, including one that might be lethal to the organism. In the absence of GAL4 the toxic target gene is silent, and is only activated in progeny arising from a cross to a GAL4-expressing line. If a protein is required in a number of developmental processes, or acts at several times in development, its separate roles can be conveniently studied by restricting ectopic expression to specific cells or tissues, or to a particular stage of development.

GAL4 can direct expression in a wide range of embry-

onic patterns in *Drosophila*, and does not appear to be excluded from particular embryonic cells or tissues. We have observed expression in cells derived from all three germ layers: the endoderm, from which the midgut derives, the ectoderm, from which the nervous system and the epidermis arise, and the mesoderm. The one tissue where we have not been able to detect GAL4 mediated expression is the female germ line. In enhancer detection/*lacZ* screens, about one third of the insertion lines express  $\beta$ -galactosidase in the germ line (Fasano and Kerridge, 1988). In contrast, none of the enhancer detection/GAL4 lines we screened show appreciable expression in the germ line (A.B. and N.P., unpublished data; D. McKearin, personal communication). GAL4 translation might be selectively repressed in the germ line, or the GAL4 message may be selectively degraded. Alternatively, GAL4 may activate transcription in conjunction with another protein that is not present in germ cells.

The earliest that we are able to detect GAL4-directed expression in embryos, by staining with antibodies against either  $\beta$ -galactosidase or *even-skipped*, is after the completion of gastrulation, between 3.5 and 4 hours of development. A GAL4 insertion at the *hairy* locus can direct expression in a pattern that resembles *hairy*, but only from about stage 8 (3:10 to 3:40 hours; Campos-Ortega and Hartenstein, 1985), an hour or so after the onset of stripes of *hairy* protein expression. Whereas GAL4 mRNA can be detected at the cellular blastoderm stage (K. Staehling-Hampton and F. M. Hoffmann, personal communication) transcription of the target gene is not seen until three and a half to four hours of development. Two possible explanations for the delay in activation in early embryos are: (1) that the GAL4 mRNA is not translated during early embryonic development, or (2) that GAL4-mediated transcriptional activation requires a co-activator, which is itself not expressed until three to four hours after fertilization. These alternative explanations will be distinguishable when antibodies that recognize GAL4 in *Drosophila* embryos become available.

### Expression of *even-skipped* in *hairy* stripes alters epidermal cell fate

The segmentation gene *even-skipped* encodes a homeodomain protein that directs cell fates in the ectoderm and in the central nervous system (Frasch et al., 1987, 1988; Doe et al., 1988). Each of these roles can be studied independently using the GAL4 system to target *even-skipped* expression. We have misexpressed *even-skipped* in the *hairy* and *paired* expression patterns, expanding the *even-skipped* stripes anteriorly into the even-numbered parasegments, and repressing *wingless* in those cells that ectopically express *even-skipped*. This ectopic expression alters the fate of cells that would normally secrete naked cuticle, such that they now secrete denticles. Since *wingless* is required for the secretion of naked cuticle (Bejsovec and Martinez Arias, 1991; Dougan and DiNardo, 1992), it is possible that the ectopic denticles we observe are a direct result of *even-skipped* repressing *wingless* in the posterior cells of the even-numbered parasegments.

We found cuticle defects in the embryos that ectopically express *even-skipped*, but observed no associated central

nervous system defects. We have performed reciprocal experiments in which we have been able to disrupt CNS development without affecting the cuticle (A.B. and N.P., unpublished data). GAL4-dependent targeted expression can thus be used effectively to study the roles of *even-skipped* in either epidermal or central nervous system development.

#### Directed expression of activated *Dras2* to generate adult phenotypes for genetic analysis

The GAL4 system can be used to restrict the expression of lethal products, and in so doing, to generate dominant adult phenotypes for use in genetic screens. We have targeted expression of an activated form of the *Drosophila* Ras homologue, *Dras2*. Using a randomly integrated GAL4 gene to drive transcription, we have been able to target expression of *Dras2<sup>Val14</sup>* to the eye and wing imaginal discs, and to generate flies exhibiting a rough eye phenotype and wing vein defects, but normal viability and fertility. These mutants can now be used to identify other proteins in the *Dras2* signal transduction pathway by isolating second-site mutations that enhance or suppress the *Dras2<sup>Val14</sup>* phenotype. Furthermore, possible epistatic relationships can be tested by combining the *Dras2<sup>Val14</sup>* mutations with mutations that affect eye or wing development, such as EGF receptor mutations (Baker and Rubin, 1989; Clifford and Schupbach, 1989).

#### Future prospects

In conclusion, we have developed a two-part activation system that allows the restricted expression of any gene of interest. We have utilized a transcriptional activator that can activate transcription in yeast, in *Drosophila*, in plants and in mammalian cells, such that the enhancer detection/GAL4 system we describe might be extended for use in other organisms. For example, enhancer- or promoter-detection/*lacZ* constructs have been developed for use in mice (Allen et al., 1988; Gossler et al., 1989), and could be modified to express GAL4.

GAL4 can be used to misexpress regulatory proteins, or to target expression of mutated proteins, producing either gain of function or dominant negative phenotypes. Mutant proteins from other species might be expressed in *Drosophila* to generate phenotypes for use in genetic screens. As an example, we have expressed an activated form of the human *c-raf1* gene in flies, generating embryonic and adult phenotypes consistent with the known roles in development of the *Drosophila raf* protein kinase, as well uncovering previously unidentified functions (A.B. and N.P., unpublished data).

The GAL4 system allows the expression of genes encoding toxic products, such as activated oncogenes. More recently we have used GAL4 to target expression of the A chain of diphtheria toxin, and have demonstrated GAL4-dependent cell killing (A.B., J. Haseloff, H. M. Goodman and N.P., unpublished data). It is now possible to kill any cell expressing GAL4 and to study the consequences of cell- and tissue-specific ablation on embryonic, larval or adult development. For example, targeted killing within the nervous system may result in reproducible behavioral

abnormalities, such that the role of particular cells in generating behavior can be analyzed.

Finally, other components of the GAL regulatory pathway, which has been so thoroughly analyzed in yeast, could be imported into flies to diversify further the GAL4 system. For example, a temperature sensitive allele of GAL4 (Matsumoto et al., 1978) has been described that, if expressed in flies, would allow ectopic expression to be restricted temporally as well as spatially. Alternatively, GAL4 could be used to drive expression of target genes encoding temperature sensitive proteins. To refine further the pattern of GAL4-dependent transcription, a negative regulator of GAL4, the GAL80 protein, might be introduced into *Drosophila* and expressed in a pattern that overlaps that of GAL4. A temperature sensitive allele of GAL80 has also been described (Matsumoto et al., 1978).

The level of GAL4 induced expression can be modulated by increasing or decreasing the number of GAL4 binding sites upstream of the target gene, or by using GAL4 derivatives, or activators fused to the GAL4 DNA binding domain, that are capable of activating transcription to different degrees (Ma and Ptashne, 1987a,b; Gill and Ptashne, 1987; Johnston and Dover, 1988). We have constructed an enhancer detection vector with GAL4 derivative 236 (Ma and Ptashne, 1987a), which is about half as active in yeast as full length GAL4, and have found it to activate transcription in flies (A.B. and N.P., unpublished data). While we have exclusively discussed the activation of target genes with GAL4, it may also be possible to repress the expression of endogenous genes by using GAL4 to drive the transcription of antisense RNAs. GAL4 can activate and maintain transcription at high levels, which might improve the efficiency of sense/antisense interactions.

We would like to thank Beth Noll and Marcy Engelstein for technical help with the enhancer detection screen and the assays for embryonic  $\beta$ -galactosidase expression, and Robin Pinto of the scanning electron microscope facility at the Museum of Comparative Zoology, Harvard University, for instruction in preparing samples for SEM, and for producing the micrographs shown in Fig. 7. For kindly providing DNAs and flies, we thank Janice Fischer-Vize, Konrad Basler, John Bishop, Victor Corces, Claude Desplan, Laurent Fasano, Ed Giniger, Phil Ingham, Liam Keegan, Mike Levine, Gerry Rubin and Rahul Warrior and for invaluable vectors, we thank Doug Knipple, Hugo Bellen, Clive Wilson, Walter Gehring, Carl Thummel and Vincent Pirrotta. For communicating results prior to publication we thank Dennis McKearin, Karen Stachling-Hampton, Mike Hoffmann and Nipam Patel. We are most grateful for the repeated gifts of antibodies from Nipam Patel and Bob Holmgren. We thank Rich Baldarelli, Joe Duffy, Jim Haseloff, Bob Holmgren and Michele Musacchio for fruitful discussions and/or comments on the manuscript. A.B. was supported by postdoctoral fellowships from the Helen Hay Whitney Foundation and the NIH, and is a Leukemia Society of America Special Fellow. This work was supported by the Howard Hughes Medical Institute.

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(Accepted 16 March 1993)



Associate editor: P.K. Chiang

## Model systems in drug discovery: chemical genetics meets genomics

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### Abstract

Animal model systems are an intricate part of the discovery and development of new medicines. The sequencing of not only the human genome but also those of the various pathogenic bacteria, the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila*, and the mouse has enabled the discovery of new drug targets to push forward at an unprecedented pace. The knowledge and tools in these “model” systems are allowing researchers to carry out experiments more efficiently and are uncovering previously hidden biological connections. While the history of bacteria, yeast, and mice in drug discovery are long, their roles are ever evolving. In contrast, the history of *Drosophila* and *C. elegans* at pharmaceutical companies is short. We will briefly review the historic role of each model organism in drug discovery and then update the readers as to the abilities and liabilities of each model within the context of drug development.

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**Keywords:** *Caenorhabditis elegans*; *Drosophila*; Mouse; Yeast; Drug discovery; Genomics; Genetics; Mechanism of action; Microarray; RNA interference

**Abbreviations:** AD, Alzheimer's disease; EST, expressed sequence tag; GOF, gain of function; LOF, loss of function; PCR, polymerase chain reaction; RNAi, RNA interference.

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## 1. Introduction

Drug discovery has evolved over the years mostly due to the development of new technologies combined with a less simplistic view and better understanding of biology. Drugs

were once developed through a trial-and-error process where combinations of plant or animal extracts were experimented with in vivo on human subjects. While such trials may have shown some therapeutic benefit, they most often resulted in no effect or adverse effects. Through these types of experi-

ments, rudimentary medicinal knowledge was obtained. As technology advanced, complex mixtures of chemicals from extracts were separated and the individual components were tested for therapeutic value. In addition, animal models, such as rats, pigs, dogs, and mice, were harnessed to test the effects of isolated compounds before they were tried in humans. Bacteria and yeast were utilized in the drug discovery process, mainly as a method of producing complex organic molecules in large quantities for testing. Drug discovery took yet another turn with the advent of molecular biology and combinatorial chemistry. The combination of these technologies allowed for the development of specific compounds that bound to and affected the activity of individual proteins. The challenge in drug discovery was to choose the correct protein to target with compounds. The advancement of genetics in yeast, *Caenorhabditis elegans*, and *Drosophila* and the recognition that many biological mechanisms and protein functions have been highly conserved has led to their more recent integration into the drug discovery process. Bacteria, yeast, *C. elegans*, *Drosophila*, and mouse models all have a unique utility and history in drug discovery, and the combination of these systems is helping to drive the selection and validation of tomorrow's small molecule medicines.

## 2. Bacteria

### 2.1. Brief history

Bacteria have long been the subject of scientific study due to their ability to cause disease in humans (Lederberg, 2000). One of the major advances in the health and well-being of human civilizations was the development of the antibiotic. While the introduction of antibiotics had an enormous impact on the ability to treat bacterial infections, they continue to be the leading cause of death worldwide (Binder et al., 1999). Moreover, the effectiveness of antibiotics has been eroded by the appearance of resistance to nearly all classes of antibiotics among bacterial pathogens, coupled with the fact that essentially only one new class of antibiotics has been introduced by the pharmaceutical industry since the mid-1970s. Clearly, the discovery of new therapies against the diseases caused by bacterial pathogens is a critical need of the 21st century. Over the past decade, the field of genomics has revolutionized both the basic research in general and the pharmaceutical industry in particular. This section of the review will focus on the impact of genomics on the study of bacteria and the development of therapies to fight bacterial infections.

Microscopes were invented in the late 1600s by Leeuwenhoek and Hooke, but handmade instruments were used primarily for observing and classifying microorganisms rather than being put to use investigating the role of bacterial pathogens in diseases of humans and animals. This changed following the refinement and acceptance of the

scientific/experimental method over the next two centuries and the rapid technological advances of the Industrial Revolution. Some initial observations of microbes in the blood of infected hosts were made in the mid-1800s, but it was not until the development of pure culturing techniques by Lister and Koch and the establishment of Koch's postulates for etiologic proof that a specific agent causes a certain disease that microbiology entered its Golden Age. During this time (the last 2 decades of the 19th century), nearly all major bacterial disease agents were described, such as *Bacillus anthracis* (anthrax), *Salmonella typhi* (typhoid fever), and *Corynebacterium diphtheriae* (diphtheria), to name a few. These pioneering microbiological studies also lead to the development of a number of therapeutic vaccines for infectious diseases.

From the Golden Age of Microbiology to the present, microbiological research continued to contribute to knowledge about infectious diseases and, just as importantly, has spurred advances in other scientific fields, such as immunology, chemotherapeutic treatment of disease, and genetics. The influence on immunology was mainly centered on the study of the immune response to vaccination—the increased resistance was explained by Metchnikoff's cellular theory and Bordet's humoral theory, both of which are known today to be important arms of the mammalian immune response. Additionally, the specificity of the immune response was initially studied in the context of the response to bacterial antigens, such as the production of antibodies to specific types (serotypes) of bacterial capsular polysaccharides. Chemotherapeutics directed specifically against bacteria, a concept advocated by Ehrlich in the 20th century, set the stage for the modern era of antibiotics after Domagk demonstrated the antibacterial properties of the dye Prontosil in 1935. The serendipitous discovery of penicillin by Fleming in 1928, and subsequent demonstration of its utility by Florey and Chain during World War II, is another example of how basic microbiological research was translated into new drugs by the then-developing pharmaceutical industry (Henderson, 1997).

The field of genetics was also fundamentally affected by bacterial genetic research. Studies on transformation of the pneumococcus by Griffith (1928) and Avery et al. (1944) established a critical concept in genetics: that DNA was the genetic material of life. This was a controversial concept at that time but was accepted by the time of Hershey and Chase's (1952) report. Over the next few decades, many genetic breakthroughs—determining the mechanisms of replication, transcription, and translation, the genetic code, and the structure and expression of genes—were found through microbiological research using bacteriophage and the workhorse bacterium *Escherichia coli*. In addition, a number of molecular tools were discovered in bacteria, such as DNA ligase and restriction enzymes. However, it was not until the landmark work of Cohen et al. (1973) that these enzymes were used together, along with plasmid replicons, to enable the cloning of DNA fragments. This led to the

birth of the field of molecular biology, which in turn had a profound effect on drug discovery and development. Instead of using brute force protein purification to isolate targets for small molecule compounds or therapeutic proteins, these entities could be supplied in bulk by cloning and expression technologies. The age of molecular biology transformed the pharmaceutical industry—and the newly spawned biotechnology industry—on an unprecedented scale, perhaps only matched by the recent breakthroughs in genomics.

## 2.2. Current usage of bacterial models

One recurring theme regarding the use of bacteria is that research is done first in the simpler microbial systems before moving on to the more complex eukaryotic systems. This pattern has been often repeated, most recently with the field of genomics. Antimicrobial research was the first field to benefit from advances in genomics due to the relatively small size of microbial genomes and the existence of well-defined, genetically tractable systems. Bacteria are important tools being used for drug discovery today—some of the areas impacted are outlined in Sections 2.3–2.6.

## 2.3. Genome sequencing

The development of automated sequencers (Hunkapiller et al., 1984) and the infrastructure to support high-throughput sequencing, assembly, and data management practices enabled the publication of the first bacterial genome (Fleischmann et al., 1995). The publication of the *Haemophilus influenzae* sequence was a milestone for genome sequencing for several reasons. First, the whole genome shotgun method was used to sequence an entire genome—the accepted paradigm up to that point was a time- and resource-intensive cosmid-by-cosmid approach. Second, the project set a high bar for the publication of future genome sequences—an entire genome sequence without gaps, extensively annotated, and making use of the then-new technology of the World Wide Web to allow free access to an unprecedented volume of data. Finally, the determination of the sequence of a ~2 Mb segment of genomic DNA indicated that it was time to shift the focus of the human genome project to sequencing, as opposed to mapping. To the surprise of many, the whole genome shotgun sequencing methodology was successfully modified and used to completely sequence the ~120 Mb *Drosophila* (Adams et al., 2000) genome and was utilized, to a degree, in assembling the ~3000 Mb human (Venter et al., 2001) genome.

Microbial genome sequencing projects can range in quality from low-pass sequencing efforts, which rapidly identify the majority of genes in a genome (often seen in industrial settings), to whole genome sequencing projects published in peer-reviewed journals. Low-pass sequences are more error prone and contain significant gaps, providing an incomplete “parts list.” Complete genomes represent a firm framework for future experimental work, since the genome has become a

finite entity, with a complete list of genes and exact coordinates for each gene. Since 1995, hundreds of microbial genome projects have been initiated (Nelson et al., 2000), and to date, over 50 microbial genomes have been completed (to view current statistics, consult the following URLs: <http://www.tigr.org/tdb/mdb/mdbcomplete.html> and <http://ergo.integratedgenomics.com/GOLD>) (see Table 1). However, even with this wealth of microbial genome information from representatives all over the evolutionary tree of life, genome projects continue to result in identifying 25–35% of the genes whose function is not known. Clearly, the work is just beginning with the sequencing of genomes.

## 2.4. Bioinformatics of microorganisms

For microbial genome projects, many of the same bioinformatic techniques are used as in other systems, but there are several issues unique to bacteria. One is the different structure of genes in bacteria as compared with other organisms, and a number of computer programs exist to automate bacterial gene start site prediction (Hannenhalli et al., 1999; Suzek et al., 2001), to find genes in genome sequence data (Isono et al., 1994; Salzberg et al., 1998; Badger & Olsen, 1999), and to address the issue of operons (Overbeek et al., 1999; Snel et al., 2000; Ermolaeva et al., 2001).

Another bioinformatic tool originally developed to help analyze bacteria genome data are programs that perform whole genome comparisons. The Microbial Genome Concordance tool (Brucoleri et al., 1998) allows users to identify a concordance of genes conserved among selected organisms at any selected similarity level and allows other genomes to be subtracted out. This program was designed to allow researchers to build in both spectrum and selectivity for novel classes of antibiotics by identifying potential drug targets (gene products) that are conserved in certain groups of bacteria but not found in humans.

Prior to the publication of microbial genome sequences, much of the phylogenetic classification performed with bacteria focused on taxonomic classification or establishment of homology among genes. With the current availability of bacterial genome information, hundreds of protein-protein interactions can be predicted based solely on sequence comparisons (Enright et al., 1999). Moreover, genome sequence information can be coupled with other experimental data (structures, domain shuffling, expression patterns, and gene adjacency in genomes) to allow new computational approaches to determining gene function (Marcotte et al., 1999). Finally, techniques, such as metabolic pathway modeling and cellular role categorization, were first worked out with microbial sequence data. These techniques are important since they can be used to organize and give context to the vast amounts of data that genomics technologies, such as microarraying, proteomics, and high-throughput biology techniques, generate. For example, it is possible to map differential expression data onto metabolic pathways or role categorization schemes using third-party software packages, such as

Table 1  
Published bacterial genomes (as of August 2002)

Genome	Mb	Publication	Genome	Mb	Publication
<i>Agrobacterium tumefaciens</i>	5.3	<i>Science</i> 294:2317–2323; 2323–2328 (2001)	<i>Mycoplasma pneumoniae</i>	0.81	<i>Nucleic Acids Research</i> 24:4420–4449 (1996)
<i>Aquifex aeolicus</i>	1.5	<i>Nature</i> 392:353 (1998)	<i>Mycoplasma pulmonis</i>	0.96	<i>Nucleic Acids Research</i> 29:2145–2153 (2001)
<i>Bacillus halodurans</i>	4.2	<i>Nucleic Acids Research</i> 28:4317–4331 (2000)	<i>Neisseria meningitidis</i> MC48 <sup>1</sup>	2.2	<i>Science</i> 287:1809–1815 (2000)
<i>Bacillus subtilis</i>	4.2	<i>Nature</i> 390:249–256 (1997)	<i>Nostoc</i> sp.	7.2	<i>DNA Research</i> 8:205–213 (2001)
<i>Brucella melitensis</i>	3.2	<i>PNAS</i> 99:9509–9514 (2002)	<i>Pasteurella multocida</i>	2.4	<i>PNAS</i> 98:3460–3465 (2001)
<i>Borrelia burgdorferi</i>	1.4	<i>Nature</i> 390:580–586 (1997)	<i>Pseudomonas aeruginosa</i>	6.3	<i>Nature</i> 406:959–964 (2000)
<i>Buchnera</i> sp.	0.64	<i>Nature</i> 407:81–86 (2000)	<i>Ralstonia solanacearum</i>	5.8	<i>Nature</i> 415:497–502 (2002)
<i>Campylobacter jejuni</i>	1.64	<i>Nature</i> 403:665–668 (2000)	<i>Rickettsia conorii</i>	1.2	<i>Science</i> 293:2093–2098 (2001)
<i>Caulobacter crescentus</i>	4.0	<i>PNAS</i> 98:4136–4141 (2001)	<i>Rickettsia prowazekii</i>	1.1	<i>Nature</i> 396:133–140 (1998)
<i>Chlamydia muridarum</i>	1.0	<i>Nucleic Acids Research</i> 28:1397–1406 (2000)	<i>Salmonella typhi</i>	4.8	<i>Nature</i> 413:848–852 (2001)
<i>Chlamydia pneumoniae</i> <sup>1</sup>	1.2	<i>Nature Genetics</i> 21:385–389 (1999)	<i>Salmonella typhimurium</i>	4.8	<i>Nature</i> 413:852–856 (2001)
<i>Chlamydia trachomatis</i>	1.0	<i>Science</i> 282:754–759 (1998)	<i>Sinorhizobium meliloti</i>	6.7	<i>Science</i> 293:668–572 (2001)
<i>Chlorobium tepidum</i>	2.1	<i>PNAS</i> 99:9509–9514 (2002)	<i>Staphylococcus aureus</i> <sup>1</sup>	2.8	<i>Lancet</i> 357:1225–1240 (2001)
<i>Clostridium acetobutylicum</i>	4.1	<i>Journal of Bacteriology</i> 183:4823–4838 (2001)	<i>Streptococcus pneumoniae</i> <sup>1</sup>	2.2	<i>Science</i> 293:498–506 (2001)
<i>Clostridium perfringens</i>	3.0	<i>PNAS</i> 99:996–1001 (2002)	<i>Streptococcus pyogenes</i> <sup>1</sup>	1.8	<i>PNAS</i> 98:4658–63 (2001)
<i>Corynebacterium glutamicum</i>	3.3	S. Nakagawa, unpublished observation	<i>Streptomyces avermitilis</i>	8.7	<i>PNAS</i> 98:12215–12220 (2001)
<i>Deinococcus radiodurans</i>	3.2	<i>Science</i> 286:1571–1577 (1999)	<i>Streptomyces coelicolor</i>	8.7	<i>Nature</i> 417:141–147 (2002)
<i>Escherichia coli</i> <sup>1</sup>	4.6	<i>Science</i> 277:1453–1474 (1997)	<i>Synechocystis</i> sp.	3.5	<i>DNA Research</i> 3:109–136 (1996)
<i>Fusobacterium nucleatum</i>	2.1	<i>Journal of Bacteriology</i> 184:2005–2018 (2002)	<i>Thermoanaerobacter tengcongensis</i>	2.6	<i>Genome Research</i> 12:689–700 (2002)
<i>Haemophilus influenzae</i>	1.8	<i>Science</i> 269:496–512 (1995)	<i>Thermosynechococcus elongatus</i>	2.5	<i>DNA Research</i> (2002) In press
<i>Helicobacter pylori</i> <sup>1</sup>	1.6	<i>Nature</i> 388:539–547 (1997)	<i>Thermotoga maritima</i>	1.8	<i>Nature</i> 399:323–329 (1999)
<i>Lactococcus lactis</i>	2.3	<i>Genome Research</i> 11:731–753 (2001)	<i>Treponema pallidum</i>	1.1	<i>Science</i> 281:375–388 (1998)
<i>Listeria innocua</i>	3.0	<i>Science</i> 294:849–852 (2001)	<i>Ureaplasma urealyticum</i>	0.75	<i>Nature</i> 407:757–762 (2000)
<i>Listeria monocytogenes</i>	2.9	<i>Science</i> 294:849–852 (2001)	<i>Vibrio cholerae</i>	4.0	<i>Nature</i> 406:477–483 (2000)
<i>Mesorhizobium loti</i>	7.5	<i>DNA Research</i> 7:331–338 (2000)	<i>Xanthomonas axonopodis</i> pv. <i>citri</i>	5.1	<i>Nature</i> 417:459–463 (2002)
<i>Mycobacterium leprae</i>	3.2	<i>Nature</i> 409:1007–1011 (2001)	<i>Xanthomonas campestris</i>	5.0	<i>Nature</i> 417:459–463 (2002)
<i>Mycobacterium tuberculosis</i> <sup>1</sup>	4.0	<i>Nature</i> 393:537 (1998)	<i>Xylella fastidiosa</i>	2.6	<i>Nature</i> 406:151–157 (2000)
<i>Mycoplasma genitalium</i>	0.58	<i>Science</i> 270:397–403 (1995)	<i>Yersinia pestis</i> <sup>1</sup>	4.6	<i>Nature</i> 413:523–527 (2001)

<sup>1</sup> Multiple genomes completed for this organism since original publication.

Pathway Tools from Doubletwise (Marcotte et al., 1999), GeneSpring from Silicon Genetics (<http://www.sigenetics.com>), or Resolver from Rosetta (<http://www.rosettatabio.com/products/resolver>). Metabolic pathway modeling has resulted in some excellent knowledge bases, such as the EcoCyc Encyclopedia of *E. coli* Genes and Metabolism (<http://www.ecocyc.org>) (Karp et al., 1996). An example that demonstrates the power of metabolic pathway analysis and the utility of having complete genome sequence data is a study examining the presence/absence and conservation of citric acid cycle enzymes across 19 microbial genomes (Huynen et al., 1999). Role categorization methods have also been developed for organizing microbial genome data, and many were modified from the scheme developed by Riley (1993). This scheme, along with the Gene Ontology effort that has been used primarily in model organism sequencing projects (Ashburner et al., 2000), is being combined into a single role classification effort known as the Gene Ontology Consortium (<http://www.geneontology.org>).

## 2.5. Modern experimental techniques

A number of genomics technologies exist for the analysis of all genes and/or gene products in a genome. Although these tools are being used for all of the steps in the drug discovery and development process, much of the effort in bacterial research is focused on the use of these technologies for antibiotic target identification and validation.

### 2.5.1. Microarrays

DNA microarraying (Pease et al., 1994; Schena et al., 1995) is a high-throughput transcriptional profiling technique increasingly used to examine differential gene expression in bacteria. Many experiments have been performed in industrial settings, and these drug discovery-focused studies are beginning to be published in peer-reviewed journals. One study focused on drug mechanism of action involves the pathogen-causing tuberculosis and the mechanism of action of the commonly prescribed antitubercular drug isoniazid (Wilson et al., 1999). Isoniazid-treated cultures

of *Mycobacterium tuberculosis* were analyzed using microarrays, and genes observed to change in response to the drug included those known to be part of the isoniazid mechanism of action as well as additional genes of unknown function that may be involved in as yet recognized important cell pathways. A second study used microarrays to investigate the role of 2 key regulators of virulence in the Gram-positive pathogen *Staphylococcus aureus* in an effort to investigate the development of treatments against pathogenesis-based targets (Dunman et al., 2001). These pharmaceutical researchers found genes known or expected to be regulated by the regulators *agr* and *SarA* but also found known genes not thought to be directly involved in staphylococcal pathogenesis, suggesting that these global transcriptional regulators play a broader role than previously realized. Finally, a number of microarray studies are focusing on the important area of host-pathogen interaction, an area of rapid advances, given the release of a number of eukaryotic genome sequences (Kato-Maeda et al., 2001).

DNA microarrays are also being put to use for genomic experiments such as whole-genome DNA-DNA hybridizations. Examples of these studies that focus on important pathogens include comparisons of BCG vaccine strains with other mycobacterial isolates (Behr et al., 1999), comparison of *Streptococcus pneumoniae* isolates with every gene in the sequenced type 4 strain (Tettelin et al., 2001), and comparison of divergent lineages of *S. aureus* with the COL genome to identify pathogenicity islands and dispensable genetic material, estimated to be ~22% of the genome (Fitzgerald et al., 2001). Additionally, non-microarray transcriptional profiling techniques can be used to look at differential gene expression in microbes. Some techniques that have found usage include differential display (Sturtevant, 2000), subtractive hybridization (Akopyants et al., 1998), and quantitative polymerase chain reaction (PCR) (Freeman et al., 1999). Quantitative reverse transcription-PCR has been used increasingly as the method of choice to validate microarray results, and the technique has become robust and accurate enough to be used as a transcriptional profiling tool in its own right. For any transcriptional profiling technique using bacterial RNA, a notable experimental distinction is the absence of a usable poly-A tail (Sarkar, 1996), requiring the modification of many of the molecular techniques that have been devised using eukaryotic RNA.

### 2.5.2. Proteomics

Proteomics technology is well suited to the study of bacteria, given the low complexity of the protein complement of a typical bacterial genome. Typical experiments involve the separation of different samples of proteins followed by observation of the changes in expression and identification of the selected proteins. The main protein separation technology is that which has been in use for the last several decades: two-dimensional SDS-PAGE (VanBogelen et al., 1999). However, it should be mentioned that newer techniques utilizing multidimensional liquid chro-

matography are being developed for “shotgun proteomics” studies (Wolters et al., 2001). For identification of protein species separated by gels, again, many of the reagents have been in use for a long time, such as Coomassie blue and silver stains, but newer reagents such as SYPRO dyes (Yan et al., 2000) are also being incorporated into proteomics experiments. Once proteins are separated and species representing differentially expressed proteins are found, the final step is protein identification, which can be done by techniques such as mass spectroscopy (MS), MALDI-TOF, and peptide sequencing (Yates, 2000). Extensive cataloging of microbial proteome components has been performed, such as a study of the *H. influenzae* proteome published 2 years after the genome sequence was determined (Link et al., 1997) or the extensive proteomic work done over decades by the Neidhardt group (VanBogelen et al., 1997).

A number of additional high-throughput biological techniques are being used to determine the function of genes discovered by microbial genome sequencing projects. One technique is the analysis of protein-protein interactions using genetic systems based on the yeast two-hybrid system (Fields & Song, 1989). Recent genome-wide protein-protein interaction studies have been performed for phage pathogens (Bartel et al., 1996) as well as for bacterial pathogens such as *Helicobacter pylori* (Rain et al., 2001). The dovetailing of this technology with oligonucleotide microarrays should bring increased throughput to genome-wide protein interaction studies (Cho et al., 1998). A second technique widely employed for research with pathogenic bacteria is global gene knockout campaigns. These have been pursued using transposon mutagenesis (Akerley et al., 1998), random (Lee et al., 1999b) and directed gene knockouts (Arigoni et al., 1998), and antisense technology (Ji et al., 2001). Additionally, it is also possible to include regulatable promoters, gene fusions, or oligonucleotide tags (Vagner et al., 1998) when performing gene knockouts, allowing for large-scale systematic analyses of gene function. Knockouts of every gene in model microorganisms such as *Bacillus subtilis* (Ogasawara, 2000) have been performed by the consortia put in place to sequence these genomes. Furthermore, techniques that analyze bacterial virulence, such as in vivo expression technology and signature-tagged mutagenesis (Chiang et al., 1999), have relied on fairly random approaches in the past. These techniques will now benefit tremendously from completed genomes and the characterization of systematic knockouts. Microbial expression systems are also currently of importance for both reliable, large-scale expression of individual genes identified by genome sequencing projects and high-throughput expression efforts, which are often used to identify new antigens from bacterial genomes for incorporation into vaccines (Wizemann et al., 2001) or for structural genomics studies (Kim, 2000). Finally, advances in screening techniques have led to a number of techniques that should assist in the discovery of new antibiotics. Termed “gene-to-screen”



technologies, many of these techniques are based on thermal denaturation, NMR, and phage display technologies and have been developed by biotechnology companies in response to the explosion in genomic information and the subsequent bottleneck in screening gene products of unknown function (reviewed by Cockett et al., 2000). Much of the work using this technology for antimicrobial drug discovery is proprietary at present, although there are some examples of recently published gene-to-screen work using phage display to identify novel antibiotics (Hyde-DeRuysscher et al., 2000; Christensen et al., 2001).

### 2.6. Differences the genome has made

Unlike other model systems, there is no one particular genome that reflects all bacteria. The first bacterial genome sequenced was *H. influenzae*, but a wealth of biochemical, physiological, and genetic data bacteria has been obtained using the Gram-negative model organism *E. coli* (Riley & Serres, 2000). Industrially important bacteria include the Gram-positive *B. subtilis* (Devine, 1995) as well as the numerous strains of *Streptomyces* that produce the antibiotics and other secondary metabolites critical to the drug industry (Chater, 1999). For infectious disease research, it can be argued that there are numerous pathogens, the most important of which have nearly all been completely sequenced. There are a number of differences that the publication of over 50 complete genomes has brought to the field of bacterial research. Genomics has helped change the face of microbiology within a decade from a gene-by-gene approach to a more comprehensive “systems biology” approach (Ideker et al., 2001). While the completion of so many genomes in a span of 6 years is a highly significant achievement, the post-genomic effort now building on this infrastructure has even more potential to change microbiology as we know it, using both computational and laboratory-based high-throughput approaches to understand gene function in the context of microbial physiology. From an infectious disease perspective, the availability of microbial genome sequences and the means to rapidly make use of it will allow an unprecedented ability to develop new therapies, such as new antibacterial small-molecule compounds (Allsop, 1998; Gray & Keck, 1999; Read et al., 2001) and new generations of vaccines (Pizza et al., 2000). With the publication of the genomes of model eukaryotes and humans, a powerful knowledge base leveraging microbial pathogenesis and cell biology work should enable the development of new-generation therapies in the near future. These therapies will target currently unexploited functions in the microbial cell such as virulence (Alksne & Projan, 2000) or that address infection from the host perspective. These new-generation therapies may improve on some the limitations of currently employed therapies and hopefully will address the problem of pathogens resistant to antibiotics (Mazel & Davies, 1999).

## 3. Yeast

### 3.1. Yeast as a research organism

*Saccharomyces cerevisiae*, commonly known as baker's, brewer's, or budding yeast, has been the subject of study for over 100 years. The species has many features that endear it to scientists. Unlike a mouse, it produces a pleasing odor and can be left in a refrigerator or freezer for many months without complaint. It is nonpathogenic and divides every 2 hours, growing as dispersed cells in liquid or as colonies on solid media, and its nutrient requirements are very economical to satisfy. It can grow either aerobically or anaerobically, depending on the carbon source provided. It can exist stably as a haploid or a diploid, and haploids can be mated and put through meiosis to recover haploid progeny in a matter of days. Although a unicellular organism, it can be induced to display such group characteristics as pseudohyphal growth, intercellular signaling, and programmed cell death. Finally, a highly versatile transformation (transfection) system has been available for several decades—linear and circular plasmids, high and low copy number, positive and negative selection systems, and regulated promoters—all these and more are possible. In addition, homologous recombination occurs with high efficiency, allowing integration of transformed DNA into chromosomes at precise locations, replacing and deleting host DNA as desired. A genetic nomenclature was adopted by consensus in 1966, and a central database maintains a list of a single accepted name (disputes are generally solved with the help of yeast-based beverages), avoiding the confusion often seen with mammalian gene names.

The yeast *Schizosaccharomyces pombe* also claims many of the assets cited above, although, in general, the molecular genetic tools available for this species have lagged. The common name “fission yeast” was given to *S. pombe* because, like most mammalian cells, it divides by fission into 2 daughter cells of equal size. *S. pombe* falls in a different genus of yeasts, and its evolutionary path probably diverged from *S. cerevisiae* nearly as long ago as the yeasts themselves diverged from the eukaryote lineage. The suffix “pombe” is derived from the yeast's discovery in a potent African beverage of that name; indeed, the 2 yeasts ostensibly have little in common apart from the ability to produce and withstand prodigious quantities of alcohol. Researchers on one type of yeast rarely have expertise in techniques for handling the other.

### 3.2. The yeast genomes

Thanks to the efforts of an international consortium, *S. cerevisiae* was the first eukaryote genome sequence to be completed (Goffeau et al., 1996). Excluding the rDNA region, this comprises 12 Mb of DNA, which is ~1/300 the size of the human genome. The number of annotated

genes fluctuates ~6000, as research continues to identify new genes and rule out old annotations (see, e.g., Kumar et al., 2002c). The genome of *S. pombe* was completed recently. It also comprises roughly 12 Mb of unique sequences and is predicted to encode just under 5000 genes (Wood et al., 2002). Comparing the predicted protein sets from the 2 yeasts and also *C. elegans*, Woods et al. also observed that 4523 *S. cerevisiae* proteins are homologous to 4050 *S. pombe* proteins, whereas 3605 *S. cerevisiae* proteins and 3281 *S. pombe* proteins are shared between all 3 organisms. Thus, these 2 yeasts have only ~15% more in common with each other than they do with a worm. The individuality of each yeast seems to arise from the remaining ~15% of their proteins, which are not shared with either of the other organisms in the comparison.

Unfortunately, no full cross-comparison between yeast and human genomes has been made since completion of the human genome sequence. Comparison between the predicted proteins of the *S. pombe* and *S. cerevisiae* genomes and 289 human disease proteins found 182 *S. cerevisiae* proteins and 172 *S. pombe* proteins with significant similarity, with ~50 of these being so close that orthology is almost a certainty (Wood et al., 2002). The proteins covered a range of human disease with effects from neurobiology to metabolism, with the largest group being those implicated in cancer. Beyond such disease genes, there is virtually complete conservation across eukaryotes within core metabolic and cytoskeletal functions. Also, in many situations where a more intensive analysis has been used, proteins thought to be absent from yeast have been found. A recent example is the identification of a caspase-type protein in yeast (Uren et al., 2000) and demonstration of its role in cell death (Madeo et al., 2002).

### 3.3. Database resources for yeast information

The community of yeast researchers numbers in tens of thousands. Coupled with the tools described in Sections 3.4–3.8, an enormous amount of genome-wide information has been generated already (Zhu & Snyder, 2002). In addition, the ability to measure and modulate so many parameters in yeast means it is a natural test bed for systems biology (Ideker et al., 2001). Gene-centric information for yeast is compiled into several public access databases that have made commendable efforts to cross-reference each other. Principal among these are the SGD based at Stanford University (Stanford, CA, USA) (Dwight et al., 2002), CYGD at MIPS-GSF (Germany) (Mewes et al., 2002), and YPD, now operated by Incyte (Palo Alto, CA, USA) (Costanzo et al., 2001). The latter is a commercial subscription database historically provided free to academic researchers and has served as a template for Incyte's Human-PSD and GPCR-PSD databases. Many other databases exist to collate specialized information in greater detail; these are indexed off the sources listed above.

### 3.4. Use of yeast as a model for drug discovery

Before genome sequencing revealed such conservation of biology, experiments using yeast were seminal to scientific acceptance that species, which diverged from the primate lineage hundreds of millions of years ago, nonetheless share basic biological processes with humans. In 1982, it was regarded as surprising when a piece of genomic DNA containing the CDC28 gene from *S. cerevisiae* was able to alleviate the phenotype of ("complement") a *cdc2* mutant of the unrelated yeast *S. pombe* (Beach et al., 1982). Nonetheless, the event went unremarked by most biologists studying mammalian systems, although, as noted above, the 2 organisms are almost as dissimilar as yeast and humans. In 1987, it was regarded as stunning when a cDNA clone from a HeLa cell library performed the same feat of complementation (Lee & Nurse, 1987). Today, the family of CDK proteins represented by that yeast mutant is the target of a number of compounds making their way through clinical trials in humans for applications from cancer to inflammation (Senderowicz, 2000). In the intervening years, yeast has been of utility to drug discovery science in a number of ways outlined below: as a surrogate for pathogenic fungi, for pathway characterization and reconstruction, in establishing compound targets and mechanism of action, and in characterizing protein function.

As can happen in the best of families, *S. cerevisiae* has a number of very unpleasant relatives among the fungi: *Candida* spp., *Aspergillus fumigatus*, and *Cryptococcus neoformans*, to name a few. Increases in numbers of immunocompromised and neutropenic patients and a broader use of implanted devices have led to increased incidence of fungal infections and the surprise emergence of Fluconazole (Diflucan, Pfizer) as a blockbuster medication. Unfortunately, current antifungal agents (amphotericin B, triazoles, and other inhibitors of sterol synthesis) are very limited in number, spectrum, and effectiveness. The molecular genetic tools available in *S. cerevisiae* have allowed its use in characterizing new azoles (Bammert & Fostel, 2000) and mechanisms of resistance to existing azoles, both theoretical (Kontoyiannis, 1999) and clinically observed (Kakeya et al., 2000). Novel antifungal drugs are an active area of research (DiDomenico, 1999) that employs yeast as a matter of routine. For example, target characterization for the sordarins, a potent but narrow-spectrum antifungal class, has utilized yeast extensively (Capa et al., 1998; Shastri et al., 2001) (for a more extensive bibliography, see review by Ma, 2001). Yeast is also contributing in the area of new-target discovery. Fungi share very few proteins with each other that they do not also share with humans, increasing the difficulty of developing broad-spectrum therapeutics without also affecting host biology. Because fungi are so adept at pumping out undesirable exogenous compounds, therapeutic dosing of an antifungal may cross into a window of inhibition for related host proteins. With the increasing availability of genome sequences for fungal pathogens,

some companies are seeking an Achilles heel in the form of some components that are unique to the microorganismal way of life. Others have chosen to prioritize candidates based on availability of background information and assays (Spaltmann et al., 1999). Much of this background information comes from studies of the nonpathogenic yeasts.

Many metabolic and signal transduction pathways are conserved throughout eukaryotes. The example of conserved cell cycle control mechanisms was cited above; thus, Novartis (Basel, Switzerland) used a yeast system to screen for Cdk4-specific antagonists (Moorthamer et al., 1998). Yeast was used to clone and characterize enzymes of the sterol synthesis pathway that represent candidates for cholesterol-lowering intervention (Robinson et al., 1993; Summers et al., 1993). After their identification as targets of the immunosuppressive agent rapamycin (Rapamune, Wyeth), the function of the Tor proteins was dissected using yeast. They are now known to be part of a conserved signaling pathway that activates eIF-4E-dependent protein synthesis (reviewed by Schmelzle & Hall, 2000). Yeast has contributed much to the analysis of MAP kinase signaling pathways, including the realization that GPCRs activate such pathways, the identification of scaffold proteins and RGS regulators (reviewed by Gutkind, 1998, 2000), and the discovery of unprecedented feedback loops (Metodiev et al., 2002). Yeast has even contributed to analysis of pathways that it lacks: the absence of endogenous receptor tyrosine kinase (RTK) signaling was exploited to allow analysis of signaling through platelet-derived growth factor- $\beta$  to phospholipase C $\gamma$ 2 (Arkinstall et al., 1995) and to investigate structure/function behavior of the SHP-2 phosphatase in the system (Arkinstall et al., 1998). In some cases, researchers have belatedly realized that a “missing” pathway does exist in yeast, thus the interpretation of extensive work dissecting the role of pro-apoptotic mammalian proteins in *S. cerevisiae* and *S. pombe* (Greenhalf et al., 1996, 1999; Clow et al., 1998; Matsuyama et al., 1999; Mandala et al., 2000; Torgler et al., 2000; Zhang et al., 2000; Zhang & Reed, 2001) is complicated by the recent discovery of a caspase-based apoptotic system in yeast (Madeo et al., 2002).

Thanks to their shared eukaryote biology, many targets for therapeutic compounds are also found in yeast. Both academic and industrial pharmaceutical research has exploited this. The mechanism of action of the topoisomerase inhibitors that are currently used as cancer chemotherapeutics was largely dissected using yeast (reviewed by Bjornsti et al., 1994). In the search for the molecular targets of immunosuppressive therapy with cyclosporine A, FK506, and rapamycin, yeast provided a means to identify primary and secondary components and to test hypotheses by stringent means such as complete removal of those components (reviewed by Cardenas et al., 1994). Yeast is being used extensively in exploration of the molecular action of the Hsp90 inhibitor Geldanamycin (e.g., Donzé et al., 2001;

Goes & Martin, 2001), a promising anticancer therapy with a complicated mechanism (Workman & Maloney, 2002).

By far, the widest use of yeast in drug discovery is in studies of protein function. These range from inferring the function a human protein, based on that of its yeast homolog, to detailed dissection of structural dependencies. Over 29 human disease genes have been cloned by functional complementation of their yeast homologs (Mushegian et al., 1997), and over 70 examples of examples of human/yeast complementation have been reported by the XREFdb project (Ploger et al., 2000). Yeast has proved successful in providing a model for the cellular defect (Pearce et al., 1999b) and even suggesting a therapeutic route (Pearce et al., 1999a) in Batten Disease, a progressive neurodegenerative disorder of a class that affects 1 in 12,500 births. There are many cases of genes of interest being cloned by structural homology and subsequently being shown to substitute for the yeast protein: 2 examples are sphingosine-1-phosphate phosphatase (SPPI), a human enzyme with a key role in interconversion of metabolites that regulate apoptosis (Mandala et al., 2000), and Chk2, the mammalian homolog of the *S. cerevisiae* Rad53 and the *S. pombe* Cds1 damage checkpoint kinases (Chaturvedi et al., 1999). Sometimes, structure/function relationships are dissected by expression of mutant forms of the human proteins: for example, after identifying SAG as a novel human protein involved in apoptosis that had a yeast homolog (Duan et al., 1999), complementation of the yeast mutant with various forms of SAG revealed domains essential for its E3 ubiquitin ligase activity (Swaroop et al., 2000). Another example of the use of yeast in structure/function analysis is provided by the human phosphoacetylglucosamine mutase genes HsAGM1 and HsAGX1 that may be involved in antibody-mediated male infertility. In each case, these proteins functionally complemented their yeast counterparts, and site-specific mutagenesis was successfully combined with in vitro and in vivo assays to identify residues essential for catalytic activity (Mio et al., 1998, 2000). Identification of likely catalytic residues to target for mutagenesis was facilitated by the extensive characterization of the hexose phosphate mutase family in yeast (Boles et al., 1994).

### 3.5. Genomic tools available in yeast

Widely used “pre-genome” techniques for manipulating yeast are covered in numerous texts. An excellent basic laboratory manual is provided by Guthrie and Fink (1991). Additionally, the “genomic tools” described in the following sections are now broadly available. These tools are relatively new, but their use will play an increasing part in disease-related research.

#### 3.5.1. Expression profiling of yeast using microarrays

Transcriptional profiling techniques allow the measurement of transcript levels for large numbers of genes in a single experiment. Yeast was the first organism for which

whole-genome arrays were available (Shalon et al., 1996) and is the model system for academic and commercial development of microarrayed probes. There are over 50 publications of yeast genome microarray experiments, including some covering as many as 119 experimental conditions (Roberts et al., 2000). In the arena of antifungal drug discovery, microarrays are being used to compare the specificity of novel agents with that of existing compounds and of mutations affecting the pathway (Bammert & Fostel, 2000). The ability to introduce a regulatable promoter in front of an essential gene and “dial down” its expression can provide the ideal surrogate for comparison with a compound directed against that target. Scientists at Rosetta (Kirkland, WA, USA) compared transcript data in such an experiment. They demonstrated that the off-target activity of a compound can be quantified by such comparisons, providing a means to group and rationally select desirable chemotypes (Marton et al., 1998).

The creation of an agglomerated public database of transcriptional profiles poses many problems of standardization, but the usefulness of such a resource for compound characterization was demonstrated by work at Acacia Biosciences, later part of Rosetta Inpharmatics. After creating a large data set of profiles from mutant strains and from compound-treated cells, they were able to use clustering algorithms to place test compounds into existing groups. Complexities of blocking a pathway such as isoprene synthesis at different steps could be revealed (Dimster-Denk et al., 1999). Rosetta also constructed a reference database of transcription profiles for 300 diverse mutations and chemical treatments in *S. cerevisiae*. As an example of the utility of such a database in compound classification, they observed that the profile for the anaesthetic dyclonine most closely resembled that of an *erg2* mutant, suggesting that this compound might mediate effects in humans by binding the  $\sigma$  receptor, the closest mammalian homolog of Erg2 (Hughes et al., 2000).

### 3.5.2. Yeast genome deletion collections

Extending the possibilities that were offered by early genomic reagents (Ross-Macdonald et al., 1999), *S. cerevisiae* research now has access to a set of strains that comprises a start-to-stop codon deletion for nearly every annotated protein coding gene in the genome (Fig. 1). For all such genes, a heterozygous diploid containing 1 wild-type copy and 1 deleted copy was created (~6000 strains). Where the function of the gene was not essential for viability under normal growth conditions, both haploid and diploid homozygous disruptants were also made (~4800 strains) (Winzler et al., 1999). Also, since the deletion construct for each gene was individually designed and constructed, 2 unique 20-base elements were inserted into the genome at the site of each deletion. The existence of these “tags,” which have been referred to as molecular barcodes, allows the identity of a particular strain to be confirmed rapidly by sequencing of a PCR product. It also

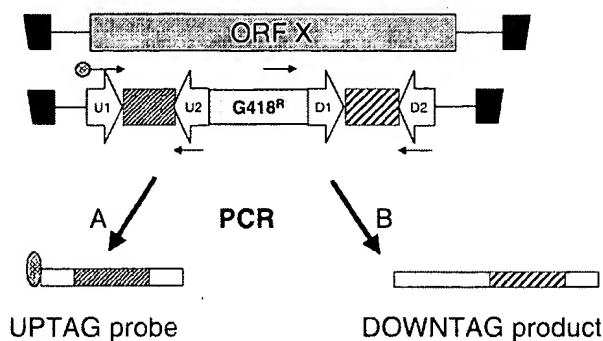


Fig. 1. Utility of features incorporated into the Yeast Genome Deletion Collection. For each protein-coding gene (ORF X in the example), a specific deletion cassette was constructed. This cassette contained flanking sequences that targeted it to replace the gene from start codon to stop codon. The marker DNA substituted for the gene confers resistance to the antibiotic G418 (G418<sup>R</sup>). At each end, it also contains unique 20-bp sequences not found elsewhere in the yeast genome. Called the “uptag” and the “downtag,” these 20-mer tags are flanked by short sequences that are common to each construct, indicated as U1, U2, D1, and D2. These common sequences can be used as priming sites for PCR, allowing every tag present in a pool to be amplified in a single reaction. (A) An amplification using primers U1 and U2, where U1 carries a molecular probe, such as biotin or a fluorophore. The resulting pool of PCR products could be hybridized to an oligonucleotide array to determine its composition. Alternatively, PCR (B) uses a primer complementary to the G418<sup>R</sup> marker region in combination with primer D2 to generate a longer PCR product suitable for sequencing. Since the downtag included in the amplified region is only present in a strain carrying a deletion of ORF X, this sequencing reaction immediately reveals the identity of the strain.

allows the presence or absence of a particular strain among a group of strains to be measured by various PCR strategies. One strategy is hybridization of a “probe” PCR product to microarrays of oligos that are complementary to the tags (Shoemaker et al., 1996). The collections have been available for less than 2 years but are already finding wide use.

### 3.5.3. Phenotypic screens

The most immediate application of the deletion collection is to direct phenotypic screening. Publications on screens for genes involved in rapamycin sensitivity (Chan et al., 2000), autophagy (Barth & Thumm, 2001), glycogen storage (Wilson et al., 2002), mitochondrial function (Dimmer et al., 2002), DNA repair (Ooi et al., 2001), proteasome inhibition (Fleming et al., 2002), and intracellular transport (Muren et al., 2001) have appeared already. The work of Ooi et al. (2001) is an example of how using a chip-based assay in combination with a pool of deletion strains can avoid thousands of individual assays.

### 3.5.4. Synthetic lethality screens

Synthetic lethality is a well-established genetic technique that has been facilitated by the deletion collections. In this approach, mutations that are individually of little or no effect are revealed to synergise and cause cell death. Historically, this was a laborious technique, but Tong et al. (2001) have now described a system for use of the

haploid deletion collection to systematically perform synthetic lethality analysis against the whole genome. This approach will find utility characterizing pathways, as it can identify redundant functions or knockon effects. It could also be used for identifying targets that would cause cytotoxicity in cancer cells that are known to lack a protein function: identification of a second protein function that is essential only in cells lacking the first protein would allow selective targeting of such cells.

### 3.5.5. Haploinsufficiency analysis

“Haploinsufficiency” screening for drug targets is a yeast genomic approach with published proof-of-principle (Giaever et al., 1999). This technique relies on the premise that a strain with reduced gene dosage for the target protein will have increased drug sensitivity. Such hypersensitive strains can be identified by individual assays on the heterozygous mutant strain collection; where compound is limiting, use of pooled strains with a chip-based readout is necessary.

### 3.5.6. Mutant gene mapping

Use of a complete collection of gene deletion mutants eliminates much of the uncertainty involved in phenotypic screens. However, point mutations historically have been the richest source of information on compound interaction. To illustrate: while complete deletion of the gene for yeast immunophilin Fpr1 results in rapamycin resistance, point mutations in the essential Tor proteins (e.g., Cafferkey et al., 1993) revealed the ultimate target. Historically, identifying the gene responsible for a mutant phenotype has been far easier in yeast than other organisms, but it was still an uncertain process. However, another way to look at the deletion collection is as a comprehensive set of mapped markers. Thus, a point mutation of interest can now be mapped by mating a haploid mutant to the ~4800 viable haploid deletion strains and then examining the haploid meiotic progeny of each such diploid for linkage between the point mutation and the G418 resistance marker in the deletion cassette. The same system applied by Tong et al. (2001) to high-throughput synthetic lethal screening can be applied here to render this process rapid and automatable.

### 3.5.7. Overexpression of yeast genes

Historically, several drug targets have been identified in yeast by virtue of the resistance granted by their overexpression (Rine et al., 1983). Overexpression analysis has been used to identify new kinases that can modulate a well-characterized MAP kinase pathway (Burchett et al., 2001). Stevenson et al. (2001) reported its use to identify new proteins implicated in cell cycle control, while Kroll et al. (1996) described synthetic lethality when a protein of interest is overexpressed in the background of an otherwise benign mutation as a method of detecting specific genetic interactions. A screen for genes whose overexpression is lethal in a proteasome-impaired mutant revealed 6 novel genes capable

of inducing apoptotic death in yeast (Ligr et al., 2001). As yet, no genome-wide reagent for systematic overexpression of yeast genes exists, although several are in construction.

### 3.5.8. Two-hybrid analysis of yeast protein interactions

Two-hybrid assays require 2 protein fusion constructs to be expressed in the same cell: if an interaction occurs between the proteins under test, it reconstitutes their attached domains into a protein with a measurable output. Performing a comprehensive analysis involves mating of a “bait” strain with 1 fusion construct to a large array of “prey” strains carrying possible interactors. Several large-scale studies of the yeast proteome have been reported to date (Fromont-Racine et al., 1997; Flores et al., 1999; Ito et al., 2000; Uetz et al., 2000). Results for yeast orthologs of human proteins are readily accessible (see database resources described above). In order to test your own “bait” protein, the Fields’ laboratory makes the complete-genome array of yeast “prey” fusion constructs described by Uetz et al. (2000) available to all interested researchers. A recent example of using yeast as a model for a target of therapeutic relevance is provided by a dissection of interactions within the 26S proteasome. Thirty-one proteasome components were individually screened as “baits” against the entire proteome, and the novel interacting components identified were then further validated by mutant analysis and reporter assays (Cagney et al., 2001).

### 3.6. Analysis of yeast protein complexes by mass spectroscopy

For protein complexes that can be physically purified, components can be identified by MS. For various reasons, the information obtained usually complements rather than replicates results from two-hybrid screens. With the first generation of CDK inhibitors currently in the clinic (Senderowicz, 2000), Honey et al. (2001) recently reported use of multiple rounds of affinity purification followed by MS to characterize components of the active yeast CDK complex tagged on its cyclin subunit. More recently, 2 commercial entities reported far larger-scale projects: Cellzome’s work included 1143 yeast orthologs of relevance to human biology (Gavin et al., 2002), while MDS Proteomics tagged 725 proteins, including a large number implicated in DNA damage responses (Ho et al., 2002). Although these projects were conducted as pilots for mammalian work on a commercial basis, they have provided a large body of freely available data for many yeast proteins with mammalian orthologs.

### 3.7. Yeast genomic reagents for biochemical analysis

Inhibition of enzyme activity is a classic therapeutic strategy: yet, for many described biochemical activities, the catalytic protein is unknown. To eliminate the onerous task of purifying such catalysts to homogeneity, Martzen et

al. (1999) created tagged fusion constructs for all yeast proteins. These can be screened for enhancement of a particular activity; they are also a useful resource for hypothesis testing with cross-linkable ligands. As a further refinement, Zhu et al. (2000) reported attachment of such tagged yeast proteins to microarrays and their screening for kinase activity and for affinity to calmodulin and phospholipids (Zhu et al., 2001). In the future, it is likely that such arrays will be used to identify molecular targets for labeled compounds of interest.

### 3.8. Yeast protein localization data

In addition to the most complete protein interaction data resources, yeast has a large volume of information on subcellular protein localization. In addition to data provided by individual studies, a genome-wide epitope tagging and immunocytochemistry project has generated subcellular localization data for nearly half the proteins in yeast (Ross-Macdonald et al., 1999; Kumar et al., 2002a), stored in a community accessible database (Kumar et al., 2002b).

While yeast has been involved in drug discovery for decades, the model system of *C. elegans* is a relative newcomer to the process. While the tools and biology of *C. elegans* are less developed, *C. elegans* is a multicellular organism and in many cases is a better model of human disease.

## 4. *Caenorhabditis elegans*

### 4.1. Brief history

From the humble beginnings of a single landmark paper published by Brenner (1974) has grown an entire field of research into the small roundworm *C. elegans*. Having been born so recently as a research tool, *C. elegans* have much less of a history and tradition in drug discovery as have the model systems of yeast, bacteria, *Drosophila*, or even mice. Yet, this unique organism is poised to make an enormous impact in the future of drug discovery. *C. elegans* was the first multicellular organism to have its genome completely sequenced and along with the bacterial genome efforts served as a proving ground for the methods that would be successfully utilized in the sequencing of the human genome. While containing a mere total of 959 somatic cells, *C. elegans* contains complex structures, such as a digestive tract, a nervous system, muscles, and complex behaviors (Horvitz & Sulston, 1990). The worm is optically clear, enabling the entire process of development to be visualized under the microscope, and is hermaphroditic, enabling genetically identical individuals to be cloned. The hermaphroditic nature of *C. elegans* also provides the means to carry out homozygous recessive and clonal screens. This allows the uncovering and mapping of recessive alleles in a timely fashion. Chemical mutagenesis and isolation of dominant

alleles can also be obtained; often, both dominant and recessive mutations can be isolated in the same screen. Many of the genetic tools and physical characteristics of *C. elegans* have been reviewed elsewhere and will not be discussed here (Plasterk, 1992; Bargmann, 1993; Avery et al., 1995; Brand, 1995; Edgley et al., 1995; Gannon & Rankin, 1995; Herman, 1995). *C. elegans* also has a rapid lifespan, with a 3- to 5-day life cycle depending on the temperature, with an individual worm being capable of producing more than 500 progeny over that period of time. Male *C. elegans* can also be produced, allowing complex genetics to be carried out in a relatively short amount of time. *C. elegans* are inexpensive to grow. With their daily diet consisting of bacteria, they can be grown both on solid substrates such as agar and in liquid culture, the latter making them amenable to higher-throughput handling appropriate for an industrial setting. The larval stages of *C. elegans* may also be frozen down, allowing the archiving of individual strains, and the entire lineage of cell divisions has been determined and is essentially invariant. As in other model systems, researchers of *C. elegans* have evolved new tools, and the completion of the genome has changed much of the way researchers in academia and in industry probe interesting biological questions, the unifying concept being that biological systems and their molecular underpinnings are now being studied as a whole.

### 4.2. *Caenorhabditis elegans* genome and conservation

A series of landmark papers published in *Science* announced the completion of the *C. elegans* genome by a multinational consortium of researchers. This consortium was responsible for developing new tools that allowed the cost of sequencing to decrease while at the same time tremendously increasing the speed by which large amounts of DNA information could be gathered, stored, and analyzed (Consortium, 1998). The first comparisons of the *C. elegans* genome with the human genome led to some striking conclusions. First, the *C. elegans* genome has approximately 19,000–20,000 genes, with only 1877 having been studied experimentally (Kim, 2001). This compares with the now estimated 30,000–40,000 human genes. The fact that a 1 mm roundworm was composed of just one third fewer genes than humans was a surprise to most scientists. In addition, preliminary analysis indicated that 74% of the human genome sequence had *C. elegans* matches (Consortium, 1998). This analysis was also aided by overlaying the genome of *C. elegans* with over 30,000 expressed sequence tags (EST) from Kohara's laboratory (Maeda & Sugimoto, 2001; Maeda et al., 2001), where they had sequenced tags representing a large number of the expressed genes in *C. elegans*. This was a monumental effort in which repeated sequencing of abundant clones from libraries was avoided by arraying and hybridizing to arrayed clones pools of multiply represented clones. A newer analysis comparing known human disease genes with the genome of *C. elegans* is revealing. In a survey of



known genes involved in human inborn errors of metabolism, 78% had easily identifiable possible *C. elegans* homologs (Kuwabara & O'Neil, 2001). This number does not take into account that DNA or amino acid alignments sometimes fail to detect conserved function and three-dimensional protein structure. Given the high conservation at the protein level between *C. elegans* and humans, it stands to follow that the function of those proteins will often also be conserved. One of the goals of genomics, in general, is to speed up the process by which functions and proper biological contexts are assigned to genes in the genome. The biology of living things is inherently complex. The fact that humans and nematodes are able to utilize similar genes in complex combinatorial ways to give rise to such divergent organisms highlights the challenge scientists face in understanding the biology. Assembling the complete gene list is a beginning, not an end. The goal is to be able to provide a complete set of functional data for all genes so that the consequences of the improper function of those genes can be quickly and easily understood and ultimately repaired when necessary. Parallel to the sequencing efforts of the *C. elegans* genome, extensive bioinformatics tools and databases were developed. Worm PD, Wormbase, Intormerator, and other databases all can be reached through a Web server (*C. elegans* www server: <http://elegans.swmed.edu/>). These tools and databases have served as a model to other genome projects on collating, coordinating, and annotating biological experiments carried out all over the world.

#### 4.3. A model for human disease and pathway identification

##### 4.3.1. Oncogenes

Although the history of *C. elegans* research is relatively short, it became apparent early that this small nematode, in some cases, would be a good model for certain human diseases. The Ras oncogene was discovered in mammals to be the homolog of the Harvey sarcoma virus *ras* gene in 1982 (Parada et al., 1982). Two groups identified a *C. elegans* version of *ras* in 1990 to be involved in vulval formation. The *C. elegans* Ras was found to be identical in 84% of its first 164 amino acids (Han & Sternberg, 1990). This discovery of a *C. elegans* “oncogene” set the stage for the use of genetics tools in *C. elegans* to define many of the genes in the Ras pathway that subsequently were found and validated in mammalian systems. In fact, many of the genes discovered in *C. elegans* to be involved in the control of cell growth via the Ras pathway are currently targets of anticancer drugs in clinic. Antibodies to the EGF receptor (Herbst et al., 2001; Kim et al., 2001) upstream of Ras, Ras farnesyltransferase (Sun et al., 1995; Norgaard et al., 1999; Lantry et al., 2000; Brown, 2001; Rose et al., 2001) inhibitors as well as inhibitors of multiple kinases downstream of Ras, are all in drug development by many companies (Dent et al., 1998; Lee et al., 1999a; Nakada et al., 2001; Segawa et al., 2001; Yacoub et al., 2001; Allen et al., 2002; Meineke et al., 2002). The identification and insight gained about

the role of these genes in Ras signaling has had a tremendous impact on the speed of drug discovery in this area. In a relatively short amount of time through the use of genetic tools, a gene of unknown function (Ras) but associated with human cancers was placed in its proper biological context, and possible targets of disease intervention were identified. *C. elegans* continues to increase our understanding of cell division, cell death, and oncology and allows experiments to be completed and questions to be answered that could not be investigated easily or speedily in humans.

##### 4.3.2. Alzheimer's disease

Other examples of the use of *C. elegans* to uncover the biological foundations of human diseases have been in the areas of cell death and Alzheimer's disease (AD). The discovery that mutations in human presenilin genes were responsible for the inheritance of early onset versions of AD in human patients was a great breakthrough (reviewed by Rohan de Silva & Patel, 1997). At the time of this discovery, however, the function and proper biological context of the presenilin proteins was a mystery. As in the case of the Ras oncogene, associating the mutation of a gene with a disease is only the beginning. Understanding the role of that gene in the context of all of the other active genes, both in normal and in diseased cells, is paramount. The findings that the human presenilin genes had *C. elegans* homologs (*sel-12* and *hop-1*) and that the human gene could substitute for the *C. elegans* gene allowed the biological function of the mutant human presenilin genes to be investigated (Levitan & Greenwald, 1995; Levitan et al., 1996). The work in *C. elegans* on the presenilin mutations not only uncovered the fact that the mutations in human presenilin were most likely less active than normal but also revealed that these genes also were involved in the passing of proper signals through the Notch signal transduction pathway. Work in mammalian systems has subsequently confirmed the connection between the human presenilins and the proper signaling of the Notch pathway (Fig. 2), and the recently discovered role

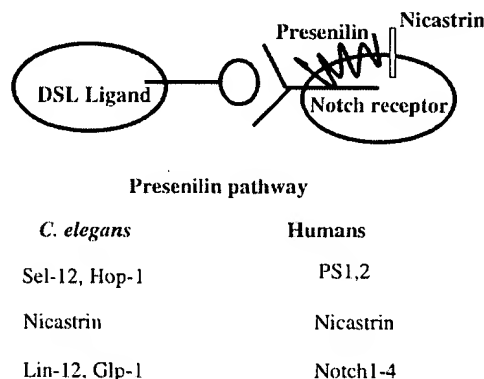


Fig. 2. *C. elegans* and human AD pathway share similar genes with similar functions.



of presenilins as novel proteases has led to the development of novel drugs against AD, which are in clinical trials (Saxena et al., 2001; Selkoe, 2001; Lai, 2002).

#### 4.3.3. Cell death

In addition to its contributions in cell growth and neurosciences, the field of apoptosis or programmed cell death has benefited tremendously from work in *C. elegans*. The first experiments to show that suicide could be the normal fate of a cell were conducted in *C. elegans* (Sulston & Horvitz, 1977; Sulston, 1983). To date, *C. elegans* genetic screens have revealed over 100 mutations that affect programmed cell death in *C. elegans* (Sulston, 1976; Hedgecock et al., 1983; Ellis & Horvitz, 1986, 1991; Ellis et al., 1991; Hengartner et al., 1992; Hengartner & Horvitz, 1994; Metzstein et al., 1996; Hengartner, 1999; Liu & Hengartner, 1999). The conservation between cell death pathways in *C. elegans* and vertebrates has allowed the field to move rapidly forward, and compounds that effect this process are under investigation in many pharmaceutical companies for their ability to modulate human diseases from cancer to neurodegeneration and stroke.

#### 4.3.4. Future utility

It is clear that in a relatively short time, *C. elegans* has had an important impact on our understanding of basic cellular mechanisms, such as cell growth and cell division. These tools have also been utilized to better understand the nervous system as well as cellular aging. *C. elegans* has only 959 somatic cells with 358 acting neurons, glial, and supporting cells. The nervous system mediates a large number of complex behaviors, including responses to mechanical and environmental stimuli. The nervous system of *C. elegans* has been entirely reconstructed from serial sections utilizing electron micrographs (Hall & Russell, 1991). The *C. elegans* nervous system utilizes many of the same neurotransmitters, such as acetylcholine, dopamine, and GABA, and *C. elegans* does respond to many of the drugs that affect the chemistry of the human brain. As the population of many countries ages, the proportion of individuals with nervous system disorders is increasing. The study of the *C. elegans* nervous system is poised to help define the biological context of genes associated with human nervous system diseases and suggests new avenues for cures in a similar fashion to the contribution of *C. elegans* to the field of cancer. Particularly intriguing is the potential use of *C. elegans* in the search for the fountain of youth.

What determines the average lifespan of one species of animals versus another? Does the degenerative nature of aging suggest that it is the result of damage rather than an innate property of the cell that determines lifespan? The genetics of *C. elegans* is allowing these questions to be interrogated in a whole-animal model. The common theories of aging range include variations in metabolic rate, oxidative damage, and caloric intake. For example, it has been

reported that the lifespan of *C. elegans* is 50% greater when grown in a food-restricted environment, and reduced oxygen levels have a similar impact on the longevity of nematodes (Klass, 1977; Honda et al., 1993). More recent studies have implicated the insulin signaling pathway and nuclear hormone receptors in *C. elegans* aging (Kenyon et al., 1993; Larsen et al., 1995). It remains to be seen whether the mechanisms that control longevity in *C. elegans* have been conserved throughout evolution. However, if one complex living organism can be modified to live twice as long through the manipulation of a single gene product, then there is a chance that pharmaceutical manipulation of a conserved gene in humans might have a similar effect.

#### 4.4. *Caenorhabditis elegans* as a model for pathogenic parasites

Four of 5 animals on Earth are nematodes (Platt, 1994), many of which are parasitic. *C. elegans* share many morphological and biochemical characteristics with their parasitic cousins and are sensitive to most of the major nematocidal drugs. The development of anthelmintic chemical screens based on the toxicity of compound decks to *C. elegans* has been widely adopted by the pharmaceutical industry yet, thus far, has met with poor success (Davis et al., 2000; Geary & Thompson, 2001). As of 1999, no new anthelmintic drugs have reached the market based upon this screening strategy (Geary et al., 1999). One of the largest problems in utilizing *C. elegans* as a model for parasitic nematodes is the lack of evolutionary conservation of parasitic species. In addition, the lack of genome sequence of parasitic nematodes has slowed comparative genomics efforts. While *C. elegans* may not be the ideal model for studying parasitic nematodes, it can be utilized as a paradigm for how one builds tools and techniques for studying parasitic nematodes. Techniques developed in *C. elegans* (see Section 4.7) are already being transferred and utilized in the study of nematode parasites. While screens in *C. elegans* for novel anthelmintic drugs have not been very successful, the use of *C. elegans* to define the mechanism of action of existing drugs and to define mechanisms of drug resistance has been of great utility. The use of *C. elegans* to define drug action is broadly applicable to drug discovery.

#### 4.5. Mechanism of action studies

Finding the molecular target that is responsible for a drug's effects is not an easy task. The use of model organisms, including *C. elegans*, in this process is clearly one beneficial approach. Utilizing the genetics of *C. elegans* to discover the molecular targets of many nematocides has clearly been successful (reviewed by Geary & Thompson, 2001). The benzimidazoles have had varying mechanisms and targets attached to them over the years, yet the studies in *C. elegans* have clearly indicated that these compounds inhibit tubulin polymerization (Driscoll et al., 1989). In a

separate example, the macrocyclic lactones were found to act through glutamate-gated chloride channels (Cully et al., 1994). In addition to nematocides, one can utilize the genetics of *C. elegans* to define the mechanism of therapeutic drugs. For instance, one recent study was able to separate 2 activities for the drug Fluoxetine (Prozac®). Fluoxetine is a known inhibitor of the serotonin reuptake system, but its complete mechanism of therapeutic action is undefined. By removing the ability of the animal to make serotonin, Choy and Thomas (1999) were able to show that Fluoxetine had additional effects besides the ability to affect serotonin levels. Genetic screens for the targets of the additional Fluoxetine effects revealed a novel class of transmembrane proteins. The ability to find all of the potential targets of a given drug is powerful. Many drugs that fail in clinical trials have strong efficacy but have intolerable side effects. In many of these cases, the side effects are due to the interaction of the compound with multiple targets, some of which are responsible for the efficacy and some for the toxicity. The use of genetic systems to tease out all of the possible targets of a compound allows the design of specific secondary screens. In addition, the use of compounds with defined targets creates the opportunity for partial loss of function (LOF) mutations. Compounds, by their nature, can be titrated; thus, the activity of a target gene can be partially turned down if complete LOF for a gene is lethal. Researchers often spend a great deal of time trying to identify hypomorphic mutation when, in fact, a compound that targets the protein could be utilized at submaximal dosage. These secondary screens enable the identification of more specific compounds that interact with and modify the beneficial targets only. The end result is less toxic, more efficacious drugs. *C. elegans* tools have become more sophisticated and faster over the last 5 years, and their application to gene function and drug discovery is just beginning to bear fruit. Novel tools for analyzing the result of loss of gene function and the ability to look at global protein/protein interactions and transcript levels on a whole genome scale will clearly be the drivers of future discoveries in *C. elegans*.

#### 4.6. Genomics tools and novel technologies

*C. elegans* was the first multicellular organism to have its complete genomic sequence completed. Analyzing the impact that this event has had on *C. elegans* research offers clues as to the progression of events in other fields. It has been an interesting evolution in both thought and research plan over the last few years, as the genomic information has had its impact. *C. elegans* scientists in the past would spend years studying a single gene product. Twenty-year scientific careers have been spent studying the intricate details of how a single receptor molecule signals. The completion of the human genome sequence has completely shifted the approach to scientific research. Whereas, in the past, a researcher might have concentrated on identifying the function of a single

receptor within a large family, one can now ask questions about the function of whole families of related receptors in the same experiment. The questions that took years to investigate now take weeks or days. However, since genomics offers an exponential increase in data volume, data collection, analysis, and interpretation is much more complex and time consuming. For example, researchers who used to be asking questions about whether a single target protein interacts with a second protein are now looking for all of the proteins in the entire organism that interact with that target protein under different conditions. The amount of data and information created from these analyses is staggering, and scientists are still struggling to organize the data in ways that are meaningful. Biological systems are very complex and change rapidly depending on conditions and time. Having genomic-based tools allows scientists to measure the complexity of changes that occur and then to try and reconstruct or model those changes. The biggest challenge going forward in all of models systems is the need to collect and organize massive amounts of data. This is, however, the only way that reasonable models that define and replicate the dynamics of living biological systems will be developed.

#### 4.7. Functional genomics

The availability of the genomic information has enabled the broader application of “reverse genetics” approaches to biological questions. Reverse genetics is a process that starts with a gene sequence and then investigates the function of that gene by removing it from an organism. In “forward genetics,” one starts from a mutation and then progresses to revealing the gene responsible. A clear example of genome impact on reverse genetics is the knockout of 20 *C. elegans* G-protein  $\alpha$ -subunits utilizing PCR, transposons, and deletion analysis (Jansen et al., 1997, 1999). In the pre-genome days, lucky researchers may have found mutations in their gene of interest, but most would have spent years analyzing a mutation and positional cloning the gene responsible. A consortium of researchers has gathered with an end goal of isolating LOF deletions for every gene in the *C. elegans* genome (<http://elegans.bcgsc.bc.ca/knockout.shtml>). The ability to know the function of every gene will be enormously useful in understanding human diseases and in identifying novel targets for drug discovery. While the increase usage of reverse genetics is leading to new discoveries, it cannot completely replace forward genetic approaches. Forward genetics screens for rare mutations that, for example, define binding pockets in enzymes, and important protein interaction sites are still very powerful. In addition, partial LOF alleles continue to be valuable in defining biological pathways where complete loss of a given gene product is lethal to the organism.

##### 4.7.1. RNA interference

RNA interference (RNAi) is a recently discovered technique through which one can ask questions about the

function of individual or groups of genes. The vast majority of genes in the *C. elegans* and human genomes do not have a function assigned to them. In *C. elegans*, injection or soaking *C. elegans* with double-stranded RNA (dsRNA) (sense and antisense) representing a given gene leads to sequence-specific LOF (Fire et al., 1998). The beauty of the technique is in its simplicity, scalability, and ease of execution. The mechanism by which dsRNA induces its effect is not entirely clear but has been conserved through evolution. RNAi is reported to function in mammalian cells (Elbashir et al., 2001), *Drosophila*, and plants. Systematic RNAi-mediated gene activation has begun to assign functional roles to over 7% of the *C. elegans* genome and is gaining steam (Grishok & Mello, 2002). The scalability of RNAi in *C. elegans* relies on several facts. *C. elegans* themselves can be grown in small volumes of liquid culture (allowing multiwell formatting). Soaking the worm or having them ingest bacteria engineered to produce dsRNA produces the intended knock-out effect, and phenotypic markers can be scored under a microscope in an automated fashion. In the context of drug discovery, the use of RNAi can aid in validating the function of potential drug targets, discovering new targets, finding the mechanism of a drug's action, and finding new entries into heavily patented pathways. In the case of mechanism of action, RNAi is used to quickly sort through and find genes whose activity is required for the drug effects. This is very similar to how one would run a forward genetic screen for resistant mutants. Because the identity of the gene is known beforehand, however, the mapping and cloning steps typified by a forward genetic screen are not necessary. How does this process work? One starts with a stack of 96-well plates, with each well containing dsRNA specific to a different gene. Worms are placed in each well containing dsRNA, and the drug in question is added and assayed for its effects on the worms. If a specific dsRNA interferes with the drug's action, the corresponding gene is probably involved in the pathway by which the drug has its effects (Fig. 3). A similar scheme is utilized to find novel genes involved in an interesting signal transduction pathway. Worms carrying a

known mutation in that pathway or drugs that are known to affect that pathway are used as a starting point, and dsRNA that interfere or enhance the pathway are quickly identified. dsRNA against different genes are able to function together, allowing for more complicated schemes and the removal of redundantly functioning genes. dsRNAi can also be utilized in the context of DNA microarrays.

#### 4.7.2. Microarrays

*C. elegans* microarray experiments have just begun to reveal interesting information. Like array technology in other fields, glass slides or chips containing a representation of the *C. elegans* genes are probed to look at mRNA expression levels under different conditions. Recent results indicate that the technology will be robust in experiments where very large differences in experimental conditions are being compared. For example, worms missing their germline have been compared with normal worms, and males have been compared with females (Piano et al., 2000; Jiang et al., 2001). In addition, microarrays are useful secondary assays for RNAi experiments and are utilized to investigate which pathways the loss of a given gene disturbs. Because the technologies for amplifying RNA from a single cell are still not mature, it remains to be seen whether transcriptional profiling will prove to be robust for answering questions about individual cell fates in *C. elegans*. The relatively small cell autonomous changes or RNA expression that occur during cell fate decisions may be difficult to identify in the context of all the cells in an animal. In addition to microarrays, the mapping of protein/protein interactions is also utilized to identify the pathways in which a given protein is actively involved.

#### 4.7.3. Proteomics

The ability to detect and analyze protein/protein interactions on a genome-wide scale has progressed tremendously in the last few years. Efforts in academic centers as well as in biotechnology companies have allowed for this technology to be automated and robust. Various platforms are available, each with the idea that if 2 proteins associate in a cell with one another they are likely to be involved in the same biological processes. In *C. elegans*, an effort to map all of the protein/protein interactions in a normal animal has been underway for several years. A group lead by Vidal has recently published several efforts towards this end in *C. elegans*. Vidal's group has mapped, via the yeast two-hybrid system, the protein-protein interactions involved in vulval development, DNA damage response, and the 26S proteasome (Kauffmann et al., 1995; Walhout et al., 2000a, 2000b; Davy et al., 2001; Boulton et al., 2002). The ability to map the interactions and define a protein "interactome" has illustrated the complex nature of cellular signaling. In addition, the interactome defined by the yeast techniques is likely to still only represent a snapshot of protein-protein interactions under a specified set of conditions. Mapping protein-protein interactions can be very informative in cases

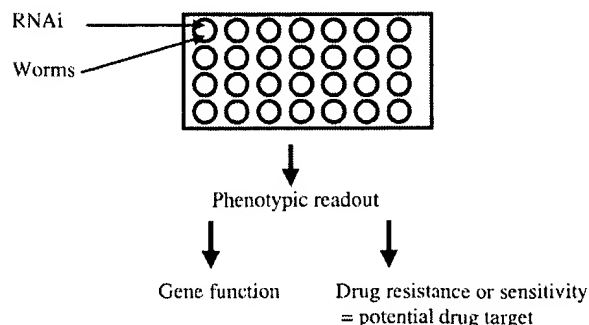


Fig. 3. RNAi in *C. elegans* is scalable and may be carried out in multiwell dishes. RNAi and worms are added to each well, respectively. The knockout effect of large numbers of genes can be observed, and if compounds are tested at the same time, the genes required for the function of the compound can be identified.

with a defined LOF phenotype. The effect of removing a particular protein from a complex can be analyzed. While *C. elegans* are a better model for some human diseases than yeast, they are not ideal for mimicking human disease. The *Drosophila* genome is a closer match to the human genome compared with *C. elegans*, and they have evolved systems, such as a basic immune system and a complex nervous system. They are poised to play an ever-increasing role in modeling human disease and drug discovery in the future.

## 5. *Drosophila* research in biomedicine

### 5.1. Introduction

For nearly 100 years, *Drosophila* genetics has been a central contributor of research on inheritance and genome organization (Rubin & Lewis, 2000). A wealth of mutant phenotypes have been discovered that disrupt developmental, physiological, and behavioral processes. Genes identified as responsible for mutant phenotypes have revealed an evolutionarily conserved architecture of cellular networks. For example, homeobox transcription factors are conserved regulators of all metazoan developmental body plans, and the combinatorial use of homeobox genes is often the difference in developing a wing or a leg. In a remarkable discovery, the mouse PAX6 eye determination gene was capable of triggering eye development when expressed in *Drosophila* (Halder et al., 1995). This confirms the existence of a common genetic program for eye specification in the developmental evolution of insect compound eyes to mammalian eye lens. In addition, genes important to human disease, such as cancer, diabetes, and inflammation, are highly conserved from *Drosophila* to humans in both structure and function. The conservation of biological processes suggest that *Drosophila* research will be an important tool for human health.

*Drosophila* genetic experiments are standard educational tools for foundations in eukaryotic genetics. There are several reasons for the popularity of *Drosophila* in research. In 1910, Morgan made the fortunate and historic decision to work on *Drosophila* based on their short life cycle and ease and cost of maintenance (Sturtevant, 1965; Rubin et al., 2000b). Within 10 years, Morgan and students, Sturtevant, Bridges, and Muller, formulated the central concepts of heredity and the first genetic maps. Fruit flies, in hindsight to Morgan, had fortuitous experimental properties of very few (4) chromosomes and “polytene” chromosomes in the salivary gland. Polytenic chromosomes have a reproducible banding pattern that reveals an unparalleled chromosomal cytology, allowing a physical map to be linked to a genetic map. Other successes of these researchers include the introduction of “balancer” chromosomes that contain chromosomal inversions to prevent recombination that allow lethal genetic mutations to be maintained in *trans* to the balancer. In 1927, Muller introduced the use of ionizing

radiation to cause genetic damage and to induce mutations, allowing chromosomal deletions, inversions, and duplications to be physically mapped on polytenic chromosomes. By 1970, an ordered set of deletions and duplications covering 70% of the *Drosophila* chromosomes were available. These tools laid the foundation of genetic screens that identified mutations with developmental phenotypes (Nusslein-Volhard & Wieschaus, 1980). Many of these genes have since been molecularly identified and are the fundamental to many multicellular signaling pathways.

Since Morgan, the fly community has continued a strong tradition of developing innovative research tools to dissect biological and genetic networks. Early *Drosophilist* tools, including the genetic and physical map, balancers, chromosomal deletions, and duplications, are still routinely used. In current times, *Drosophila* offers many versatile advantages for target discovery and validation, including genome-wide genetic mutations, genome-wide expression studies, and automated animal sorting. We will describe the convergence of traditional tools with new advances that increase the ease, speed, and value of *Drosophila* research. The major issues addressed are the (1) comparative genomics of *Drosophila* to mammalian systems, (2) genetic tools, (3) genetic screens to identify interrelated genes, (4) new genomics technologies, and (5) emerging field of *Drosophila* chemical genetics. The emphasis will be on using the *Drosophila* as a model to gap mammalian genomic sequence “information” and biological meaning.

### 5.2. Comparative genomes: are fruit flies “human”?

A new era of *Drosophila* research was announced in March 2000 with the release of the complete euchromatic *Drosophila* genome (Adams et al., 2000). This information, combined with the ability to map genes and to study function by mutation or transgenesis, allows scientists to study the relationship of genotype to phenotype. *Drosophila* genome predicts 13,601 genes as compared with ~30,000–50,000 human genes. The difference in size of human and *Drosophila* genomes may not correspond to a similar difference in the number of gene functions. Often, a single *Drosophila* gene may represent several mammalian genes. In one example among the cancer genes, a sole tumor suppressor p53 homolog was identified, and functional studies suggest that it is the likely common ancestor to mammalian p53, p63, and p73 (Ollmann et al., 2000).

Although comparative analysis of the model invertebrate organisms with humans is an evolving science, it is clear today that *Drosophila* will be a valuable model for studying human disease pathways. A simple BLAST scan shows sequence similarity to 50% between fly and mammalian proteins (>E<1010) (Papp et al., 1994; Rubin & Lewis, 2000; Rubin et al., 2000a). Thirty percent of fly genes do not appear in sequences of yeast, worms, or mammals and are suspected to be insect specific. To infer that a fly gene will have a counterpart in humans, there must be confidence

of an evolutionary relationship between these genes. Intermediate species genome information, such as the upcoming releases of the zebrafish and mice genomes, should be useful in tracing gene evolution. Without these bioinformatics “missing links,” it can be difficult to conclude the human ortholog(s) of a fly gene. Other standards utilized in the process of ortholog “calling” include conserved domain architecture or similarity across much of sequence length (Rubin et al., 2000b). Also revealing is reciprocal “BLAST” analysis, where a fly sequence is blasted against all known human sequences and then that identified human sequence is “back BLASTed” against all *Drosophila* sequences (Wälchli et al., 2000).

In a systematic analysis of human disease-associated genes, 77% are clearly related to genes in *Drosophila* (Reiter et al., 2001). Of 714 disease genes identified in the Online Mendelian Inheritance in Man (OMIM), 548 close counterparts can be found in *Drosophila*. These genes include a range of diseases implicated in cancer, cardiovascular, renal, endocrine, innate immunity, and metabolic diseases. There are general exceptions of physiology that are not preserved across species. For example, flies have no erythrocytes; therefore, hemoglobin homologs are absent. Also, genes specific to the rearrangement of immunoglobulin genes are absent in *Drosophila* that lack acquired immunity. However, invertebrates have a dozen or more cell surface proteins possessing domains resembling those found in vertebrate immunoglobulins. Examples in *Drosophila* include Dlar, Fasciclin II, fibroblast growth factor receptor-1, Frazzled, Neuroglian, and Semaphorin 2. The Ig domain in invertebrates is involved in cell-cell interactions. Thus, it is likely that the T-cell and B-cell immunoglobulin system evolved from invertebrate Ig domain proteins and the Ig domain has permitted a multitude of functions in cell communications (Rubin et al., 2000b).

There are detailed papers on fly genome orthology, but most were published before the release of the human genome. These references include comparisons of (1) disease processes, such as cancer-related genes (Potter et al., 2000), (2) biological entities, including synaptic organization (Littleton & Ganetzky, 2000), (3) cytoskeleton (Goldstein & Gunawardena, 2000), (4) cell adhesion (Hynes & Zhao, 2000), (5) cell death (Aravind et al., 2001), (6) DNA repair mechanisms (Sekelsky et al., 2000), and (7) gene families, such as nuclear hormone receptors (Enmark & Gustafsson, 2001), kinases (Morrison et al., 2000), phosphatases (Wälchli et al., 2000), and neuropeptide receptors (Vanden Broeck, 2001).

There are many examples of core *Drosophila* genes that have functionally diverged through evolution but use the same core biochemical pathway components. For example, cascades of mitogen-activated protein kinases (MAPK) transduce signals from multiple extracellular stimuli, mediating responses, such as cell proliferation, differentiation, and regulation of metabolic pathways. There are multiple MAPK in eukaryotes. In *Drosophila*, 2 such pathways have

been identified, MAPK and JNK (Stronach & Perrimon, 1999). The MAPK pathway activated by Ras is required at least 3 times during fly development and is activated by signals for specific RTK, such as the Torso receptor in the terminal system, the EGF receptor in neurogenic and wing vein pathways, and the sevenless receptor for the differentiation of photoreceptors (reviewed by Simon, 2000). Although orthologs to Torso and sevenless have not been found in mammals, the concept of cell decisions through RTK-Ras-MAPK is conserved.

Several criteria beyond bioinformatics can determine if the *Drosophila* system is a valid model to study the function of a suspected mammalian ortholog. For example, is the disease state similar in humans and fruit flies? An analogous mutation in *Drosophila* Ras to human oncogene Ras (RasV12) leads to “tumor” phenotypes when overexpressed in the *Drosophila* developing eye (Fortini et al., 1992). As detailed in Section 4.3.1, mammalian Ras research was greatly aided by genetic screens for components of a core Ras signaling pathway. This led to the connection of Ras to RTK and the identification of several novel proteins, including KSR, now a therapeutic target for cancer (Matthews & Kopczynski, 2001).

The ultimate proof to demonstrate a gene's orthologous relationship requires that a mammalian gene can rescue the LOF in the *Drosophila* ortholog (or vice versa), as described with the eyeless gene. There are now many examples of genes that work similarly across evolution (e.g., signaling components such as the human protein tyrosine phosphatase SHP-2 that rescues SHP-2 [a.k.a. *corkscrew*] mutant flies) (Perkins et al., 1996). These types of protein swaps across species have the caveat that the mammalian ortholog may not replace a loss of function (LOF) mutation due to imprecise expression. Development of routine “knockin” strategies where a mammalian gene replaces the chromosomal fly counterpart will address these issues.

Not all homologs to human gene can be found by BLAST analysis of the *Drosophila* genome but may still exist and even have similar phenotypes across species. For example, expression in *Drosophila* of human  $\alpha$ -synuclein, a protein linked to a rare form of inherited Parkinson's disease, recapitulated the specific hallmarks of Parkinson's disease, including gradual degeneration of a type of dopamine neurons (Feany & Bender, 2000). Human  $\alpha$ -synuclein, functioning in *Drosophila* even in the absence of an identified ortholog, suggests that  $\alpha$ -synuclein-like molecules exist in *Drosophila*, and current bioinformatics technologies limit ortholog identification.

### 5.3. Surfing the fly genome

Flybase (<http://flybase.harvard.edu>) is an excellent “one-stop shop” for *Drosophila* genetic-genomics resources. Flybase includes references, phenotype of mutants, gene summaries, reagents available, and links to FlyBLAST and Gadfly to identify top ortholog candidates. Gadfly contains

genomic map information as well as preprocessed “Blast” results across species. In *Gadfly*, *Drosophila* proteins are classified according to basic concepts: protein domains, molecular function, and biological process. [www.geneontology.org](http://www.geneontology.org) or “GO” attempts to classify the proteins across species using a controlled vocabulary (Ashburner et al., 2000).

In order to find the *Drosophila* cognates of human disease-related genes, Homophila (<http://homophila.sdsc.edu>) is an interspecies genomic resource linking the human and fly genomes in order to stimulate functional genomic investigations in *Drosophila* that address questions about genetic disease in humans (Reiter et al., 2001; Chien et al., 2002). Homophila provides a comprehensive linkage between the human disease genes compiled in OMIM and the complete *Drosophila* genomic sequence. Homophila is a relational database that searches based on human disease descriptions, OMIM number, human or fly gene names, and sequence similarity.

The *Drosophila* Genome Project continues to add reagents for gene analysis. This includes 240,000 EST, with the goal of obtaining sequence on all expressed genes rather than emphasis on genomic sequence “gene prediction” programs. The *Drosophila* Gene Collection (DGC) that represents 90% of the full-length clones is commercially available (Rubin et al., 2000a). In *Drosophila* research history, 3000 genes have been identified by mutation induced either by X-ray, chemical mutagenesis (EMS and ENU), or P-element transposition. The Berkeley *Drosophila* Genome Project (BDGP) will continue to generate mutation or insertions with the goal of a mutation for every gene (Spradling et al., 1999).

Also, the fly community has begun a project of mapping the protein-protein interactions encoded by the *Drosophila* genome (Stanyon & Finley, 2000). The function of most proteins involves contacting other proteins, and networks of interacting proteins control many biological processes. Discovering the interactions between proteins can provide clues as to how they function and can reveal biological regulatory pathways. The yeast two-hybrid interaction technology has been developed to enable identification of all possible binary interactions among large sets of proteins and is being applied to map protein-protein interactions encoded by the *Drosophila* genome (<http://cmmg.biosci.wayne.edu/finlab/finlabframes.html>).

#### 5.4. The *Drosophila* tool kit

In 1980, a Nobel Prize-winning experiment by Nusslien-Vollard and Weichaus undertook a systematic genome-wide mutational screen to identify all genes involved in the fundamental process of embryonic body plan. This represented the first genetic screen in a multicellular organism similar to those performed in microorganisms. This Herculean undertaking identified recessive mutations that cause defects in development. Many scientists since have mined

these hundreds of mutations, which has led to the initial characterization of many signaling pathways, including RTK, WNT, TGF, NF- $\kappa$ B, and Hedgehog—all conserved in mammals. Components of these pathways can be genetically dissected into individual components often ordered by epistasis experiments.

##### 5.4.1. Transposon mutagenesis

Over the past 20 years, molecular genetics advances have facilitated the cloning and analysis of *Drosophila* genes. Transgenic fly technology was developed using germline transformation with P-element transposons as vectors (Rubin & Spradling, 1982). Transposable P-elements are engineered so that their transposition is controlled by crossing into a fly harboring an active transposase and in the next generation crossing out the transposase to “freeze” the integrated transposon. Initially, this technology was used to test a gene (or chromosomal region) for “rescue” of a mutant phenotype. Such “gene therapy” in flies has become standard practice to correlate a gene to a mutation. The technical obstacle remains a requirement of protein expression levels at the right time and place for “rescue” of mutant phenotypes.

The stable integration of P-elements results in genetically defined mutations and has allowed development of a range of discovery platforms for gene identification and function. These include routine use of P-element vectors to ectopically overexpress an interesting gene (i.e., wild-type, mammalian ortholog, and dominant-negative mutant forms). Also, transposition is almost random, and the P-element insertion often disrupts the expression of the neighboring gene. Therefore, insertions that disrupt a gene can be identified without prediction of phenotype. In this way, P-elements can be used as a mutagen and has the great advantage of rapid identification of the flanking gene (Cooley et al., 1988). P-element-based genetic screens can sort random insertional mutations that cause specific phenotypes or to screen for gene expression patterns, a.k.a., enhancer trap screens (Bellen et al., 1989). “Enhancer trapping” employs a weak promoter that cannot normally drive gene expression in the absence of a transcriptional enhancer, coupled to a reporter. Insertion of this construct into the genome close to endogenous transcriptional enhancers activates reporter gene expression. This was an effective way to identify genes by expression pattern, such as developmentally regulated genes (Wilson et al., 1989, Fig. 5.2).

##### 5.4.2. Targeted gene disruption

Until 2000, a major limitation of *Drosophila* research was the inability to “knock out” genes systematically and efficiently. Rong and Golic (2000, 2001) discovered a clever method of gene replacement by enlisting homologous recombination. This technique requires 3 components: a site-specific yeast FLP recombinase, a transgene that expresses rare site-specific endonuclease, and a transgenic donor construct that carries recognition sites for both



enzymes and the DNA to be targeted (donor DNA). When these 3 components are expressed in a fly, the endonuclease activity creates a recombinogenic double-stranded break in the donor DNA. The FLP recombinase will catalyze the recombination between the donor DNA and the endogenous gene, leading to replacement of the endogenous gene. Rong and Golio have shown the effectiveness of this technique with several gene “knockouts” and have explored the basic principles to make it applicable to most genes (Rong et al., 2002). Technological advances in time and labor to these initial successes will make it plausible in the future to direct “knockouts” on a genome-wide scale. However, throughput will be limited by the inefficiency of injecting the germline of embryos with transgenic constructs and the screening for rare recombination events.

Another method of creating gene knockout phenotypes is the development of the post-transcriptional RNAi techniques similar to that described with *C. elegans*. RNAi is based on a process whereby specific dsRNAs induce potent and specific interference of protein expression, which, in effect, leverages the endogenous RNAi system that evolved as an antiviral/antitransposon defense mechanism of invertebrates (Tuschl, 2002). In *Drosophila*, it was first demonstrated that dsRNA directed against WNT receptors *frizzled* and *frizzled 2* injected into a developing embryo reduced the *frizzled* protein levels and phenocopied complete LOF mutants (Kennerdell & Carthew, 1998). A transgenic RNAi strategy was developed to allow gene function studies at defined stages of development and adult. This involved designing transgenic vectors to produce a hairpin-loop RNA corresponding to a gene region. This inherited transgene confers specific interference of gene expression (Kennerdell & Carthew, 2000; Piccin et al., 2001). However, many laboratories have had difficulty in routine knockdown with RNAi transgenic strategies. RNAi technologies are discussed further in Sections 4.7.1 and 5.5.1.

To analyze lethal mutations in specific situations, *Drosophila* geneticists can employ the powerful method of mosaic analysis to study small patches of homozygous mutant clones in a phenotypically normal *Drosophila*. Historically, this was used to study cell lineage mapping during *Drosophila* development by using mutants as visible cell markers (Greenspan, 1997). In modern times, it is often used to study function of genes that are homozygous lethal. Normally, mitotic recombination between chromosomes is a rare event in somatic cells, but X-rays can be used to increase the frequency of chromosomal breaks that lead to an exchange of parts between homozygous chromatids. After mitotic recombination of a heterozygous mutant, one daughter cell will be wild-type and the other daughter cell will be a homozygous mutant. When the chromosomes are “marked,” the fate of the homozygous daughter cell and its daughter cells can be followed. Today, instead of X-ray-induced mitotic recombination, a more common technique uses the yeast FLP site-specific recombinase (Fig. 4). FLP can efficiently catalyze the site-specific recombination

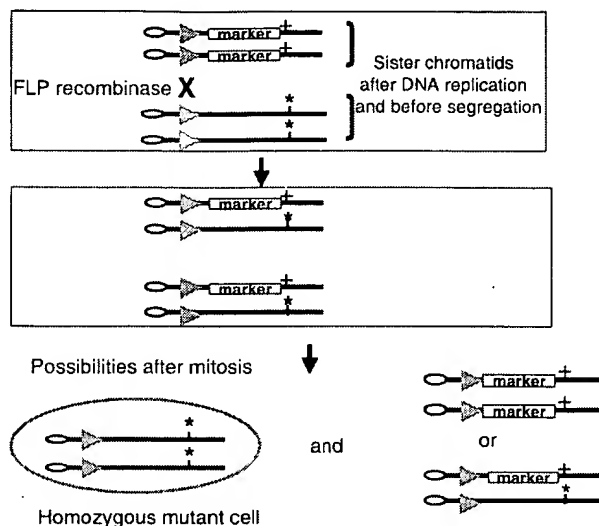


Fig. 4. Inducing mitotic recombination to generate homozygous mutant clones using the FLP/FRT system. Heterozygous mutant flies are engineered with FRT sites (gray arrowhead) oriented in the same direction and in identical positions on chromosomal homologs. Homozygotic cells are generated in heterozygotic mutant flies by inducing of FLP recombinase (heat shock or tissue-specific activation), which leads to a recombination event between the FRT sites in homologous chromosomes. Mutant alleles are followed by the lack of the “marker” gene.

between 2 FLP recombination targets (FRT) engineered into *Drosophila* (Golico & Lindquist, 1989). The key systems are the presence of FRT sequences in identical positions on both homologs and orientation in the same direction. By dictating the expression of the FLP recombinase, FRT/FLP analysis allows better temporal and spatial control than X-ray-induced recombination. In the FRT/FLP analysis, a mutant chromosome with proximal FRT sites is set up in *trans* to a chromosome with matching FRT sites and a marker gene (i.e., green fluorescent protein [GFP] or  $\beta$ -galactosidase gene, LacZ). Induction of FLP recombinase expression can be controlled by heat shock or by using the GAL/UAS binary system (described in the following section) to allow mitotic recombination at the FRT sites leading to homozygous mutant cells (reviewed by St Johnston, 2002). The FLP recombinase event will not occur in every cell allowing a random, but identifiable (lack of marker), event. Transgenes containing FRT sites are available for every chromosome arm to allow mosaic analysis of most genes.

#### 5.4.3. Ectopic expression

In *Drosophila*, two thirds of all genes have no obvious LOF phenotype (Miklos & Rubin, 1996). Therefore, gain of function (GOF) mutations generated by gene mis/overexpression can be useful to divulge function. *Drosophila* ectopic expression systems can utilize a promoter that drives either constitutive or regulated expression of the gene of interest. These constructs are prepared in a P-element vector to generate transgenic *Drosophila*. On establishing trans-



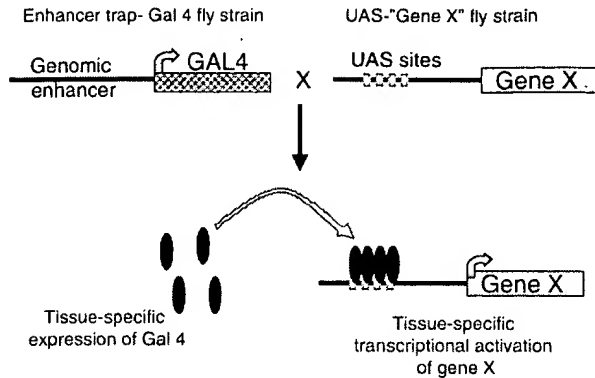


Fig. 5. Targeted ectopic gene expression using GAL4. A fly strain carrying the GAL4 gene under control of a tissue-specific genomic enhancer is crossed to a fly line carrying the targeted gene of interest, Gene X, subcloned downstream of UAS sites. The progeny of this cross will express “Gene X” in those cells and tissues in which GAL4 is present.

genic lines, ectopic expression can be studied in wild-type or mutant backgrounds. The most popular strategy in ectopic expression the last few years allows precise control of gene expression to specific cell types using the two-component GAL4-UAS inducible system (Brand & Perrimon, 1993). This system is premised on the yeast transcriptional transactivator GAL4 and its ability to activate transcription of any target gene by binding to a GAL-UAS (upstream regulating sequences) site. The 2 components are 2 different P-element transformed lines: one that expresses GAL4 lines under control of a tissue-specific promoter and the other component are flies that carry the target gene downstream of GAL4-UAS sites (Fig. 5). The progeny of a cross between promoter-GAL4 (P {GAL4}) and the GAL4-UAS lines leads to overexpression of the UAS-linked gene. The power of this system derives from versatility of the expression where a large number of possible GAL4 expression patterns are available. The P {GAL4} gene is preceded by a minimal promoter that is insufficient to drive GAL4 expression. These were generated by mobilizing the P {GAL4} element in classic P-element-mediated mutagenesis and GAL4 lines with precise tissue expression, and levels of expression are discovered by crossing to animals carrying P {UAS} with a visible reporter gene sequence, such as GFP or LacZ (Brund, 1995). If the P {GAL4} inserts near a gene, GAL4 expression is driven by genomic enhancer elements of a neighboring gene. Once the region of specificity of expression is known, any gene controlled by the UAS promoter can be expressed with that specificity by crossing the enhancer-trap GAL4 line with flies carrying the UAS transgene. In addition to ectopic expression, a new technique allows studies of LOF by using the GAL4/UAS system to restrict RNAi (Kennerdell & Carthew, 2000) in transgenic flies. Therefore, the GAL4-UAS technique is a powerful approach to study gene function as well as gain insights into the function and interactions of cells under study.

Another method for controlled ectopic expression uses FLP-mediated recombination (Struhl & Basler, 1993). In this system, a constitutive (or regulated) promoter is separated from a cloned target gene by a “FLP-out” cassette (Fig. 6). This cassette contains a transcriptional stop signal and a visible reporter gene sequence flanked by 2 FRT sites directed the same way. Upon introduction of the FLP recombinase, the FLP-out cassette sequences between the FRT sites will be removed, thus linking the constitutive promoter to the target gene and activation of transcription. This FLP recombinase event will not occur in every cell, allowing a random, but identifiable, cell (lack of marker) to overexpress the gene. New sophistications to FRT/FLP-UAS/GAL4 allow single-neuron labeling (Lee & Luo, 1999, 2001) to discover precisely neuron lineage and specification (Jeffèris et al., 2001).

A more detailed review of the above techniques can be found in Adams and Sekelsky (2002) and St Johnston (2002).

#### 5.4.4. Designing genetic screens

*Drosophila* research has developed the versatile tools needed to understand the biological function of virtually every gene. The ability to screen thousands of mutations in a specific situation is an important *unbiased* approach toward identifying new genes important to a biological function. Conventional genetic screens were performed by identifying mutations that effect processes such as viability, morphogenesis, or behavior. Later, genetic screens were developed to look for changes in specific markers of cells, such as antibodies and transgenic reporter genes (i.e., LacZ or GFP) (Plautz et al., 1996). For example, Wu and Anderson (1998) developed a bacterial-responsive LacZ assay in flies. Ethyl methane sulfonate (EMS) was used to induce mutations, and homozygous alleles were screened for the inability to respond to a bacterial infection, as measured by  $\beta$ -galactosidase activity. This screen identified several known com-

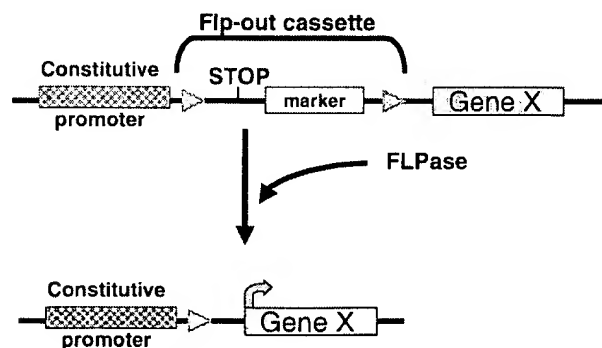


Fig. 6. FLP-out technique for targeted ectopic expression in clones of cells. The target “Gene X” is separated from a constitutive promoter by FLP-out cassette. The cassette contains a transcriptional stop signal (STOP) and a marker gene flanked by FRT sites (gray arrowheads). The FLP-out cassette prevents expression through to Gene X. Induction of FLPase leads to excision of the cassette, which will juxtapose the promoter and target gene expression. Clones expressing Gene X can be recognized by the loss of marker.

ponents of the mammalian immune response pathway, such as a NF- $\kappa$ B/Rel protein and its essential activator I $\kappa$ B (Lu et al., 2001). Therefore, novel genes from this screen may be important components of Rel protein signaling in mammalian systems, and new members of the NF- $\kappa$ B/rel pathway can be ordered by using mutants in known components (Wu et al., 2001).

Today, these types of genetics screens for chemical-induced mutations with recessive phenotypes are rarely undertaken due to the intense effort and time commitment to screen phenotypes in the third-generation and genetic mapping. As detailed in the following sections, the more common approaches to genetics screens that are transposon based, genetics modifier screens, clonal screens, or a combination of any of these techniques.

#### 5.4.5. Transposon genetic screens

In conventional genetics screens, genomes were mutagenized using chemicals or ionizing radiation to create random mutations (Greenspan, 1997). Even with the genome sequence complete and new advances in genetic mapping, identification of a chemical-induced mutation to a specific gene involves time, labor, and luck, which requires on the order of 1 scientist a year to identify 1 or 2 genes (Chen et al., 1998; Berger et al., 2001). The cost of genetic mapping can be resolved by using P-element transposition as an initial genetic screen. P-elements offer the most efficient method to generate LOF alleles on a genome-wide basis and to identify modifiers rapidly. P-element mutations are sequence “tagged,” which allows gene identification of all hits in the screen in a matter of weeks, and collections of P-element insertions lines are available for genetic screeners. There are some important disclaimers for the use of P-element mutagenesis, including that P-elements are notorious for inserting in the regulatory region of a gene, leading to a weak LOF allele that may not have a phenotype. This insertion feature is an advantage in enhancer-trap strategies and the GOF screens discussed in Section 5.5.5. A second drawback is the P-element property of insertion site restrictions (Liao et al., 2000). A pilot study suggests that only ~25% of genes allow the P-element to insert a detectable LOF phenotype (Spradling et al., 1999). New transposons, such as *mariner*, *hobo*, and *piggyBac*, need to be developed to reach a goal of strong LOF alleles for each gene (Horn & Winnier, 2000). Ideally, the fly community would greatly benefit from genome-wide screening with several types of transposons. One benefit of the chemical mutagenesis such as EMS over P-element mutagenesis is that EMS often induces single-point mutations in genes that lead to interesting dominant-negative or dominant-positive features. It is important to bear in mind that regardless of the mutagen chosen, even well-developed and extensive genetic screens will miss components. However, most genetic screens give key insights and landmark genes that hook into biological function.

In the last 10 years, dominant modifier genetics screens have become the most common approach to identify genes that influence a specific biological process. The central concept of these screens is that, if a mutation in one gene suppresses (or enhances) the phenotype of another gene, their products are likely to be directly involved in the same process (Simon et al., 1991). This approach starts with dominant phenotype related to gene, such as misexpression or overexpression. In the screening process, mutations in other genes can be crossed into starting mutant background to identify mutations that enhance or suppress the dominant phenotype. This technique can be used to test for interactions between known mutations as well as recover new mutations from de novo screens.

Ras signaling discoveries are excellent examples of the potential of *Drosophila* modifier screens translating to disease pathogenesis. Ras mutations are responsible for a significant fraction of human cancers (Lowy & Willumsen, 1993). There is a single homolog of Ras in *Drosophila*, which, like human Ras, is linked into RTK pathways. An activated Ras construct (Ras<sup>val12</sup>) containing a single-point mutation associated with human cancers that is expressed during *Drosophila* development will by-pass the require-

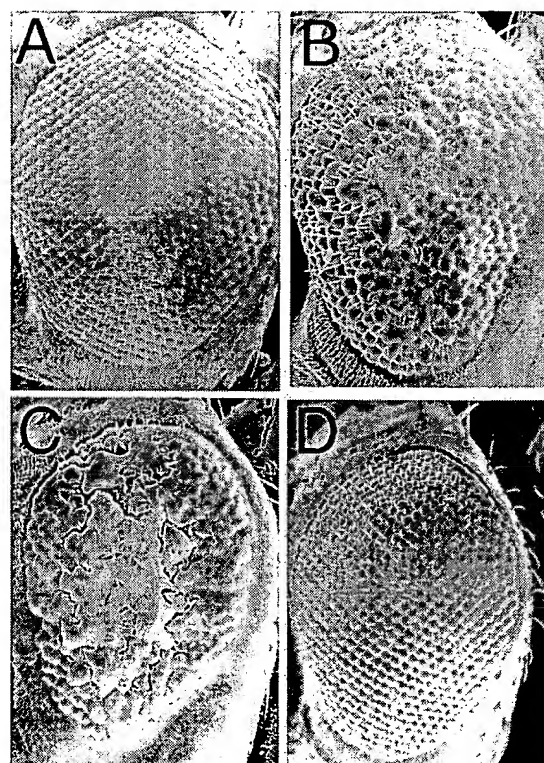


Fig. 7. Modifiers of Ras signaling identify PTP-ER and MAPK mutations. Each figure is a scanning electron micrograph of an adult *Drosophila* compound eye. (A) Wild-type. (B) Ras<sup>val12</sup> expressed with a sevenless promoter causes a rough eye (sev-Ras<sup>val12</sup>). (C) A loss of function mutation in PTP-ER enhances the sev-Ras<sup>val12</sup> phenotype. (D) A loss of function mutation in MAPK suppresses the sev-Ras<sup>val12</sup> phenotype. Data and pictures were generously provided by F. Karim. See text for references.

ment of RTK activation and will induce ectopic cell proliferation and hyperplasia (Fortini et al., 1992; Karim & Rubin, 1998). Therefore, the Ras<sup>val12</sup> phenotype is a fly model for human cancer. In a screen to understand the pathway of Ras activation in cancer, 850,000 progeny of EMS-treated flies were crossed to the dominant rough eye created by expressing activated Ras during eye development. Enhancer mutations are identified as having a rougher eye compared with Ras<sup>val12</sup> alone, and suppressors would have a smoother “wild-type eye” (Fig. 7). In this screen, LOF mutations of positive regulators of Ras signaling are expected to reduce Ras signaling and thereby scored as a suppressor of the rough eye (Fig. 7D). Conversely, LOF of negative regulators of Ras signaling are expected to increase Ras signaling and are scored as enhancers of the rough eye phenotype (Fig. 7C). This screen identified many key regulators of Ras signaling, including RAF, MEK, MAPK, 14-3-3, and several novel components, such as kinase suppressor of Ras (KSR) and PTP-ER (Therrien et al., 1995; Karim & Rubin, 1999). Mutations in KSR suppress the phenotypic effects induced by activated RAS and today is studied as an essential component of RAS signal transduction from *C. elegans* to humans. PTP-ER mutants were discovered as a novel phosphatase that enhances of Ras signaling. Therefore, its normal function is as a negative regulator of the RAS biology, and further experiments show PTP-ER to dephosphorylate and down-regulate MAPK.

The significant attribute of genetic modifier screens is the ability to identify a gene by one mutant copy. LOF of most genes are recessive, which indicates that standard heterozygous mutations with 50% gene activity are enough for wild-type function. When a process is disturbed, such as with Ras<sup>val12</sup> activity, 50% of normal function may no longer be enough to behave as wild-type. Also, screening for heterozygous phenotypes allows panning of large numbers of mutations in a single F1 generation and permits identification of lethal genes that function at multiple points of development that often are homozygous lethal. For example, a 50% reduction in KSR is associated with reduced Ras<sup>val12</sup> activity and is scored as a suppressor (Fig. 7B). Since the screens are based on ectopic phenotypes, it is important to correlate starting phenotype with gene activity. For example, mutations in Raf, a known Ras interactor, were able to suppress the Ras<sup>val12</sup>-induced rough eye, suggesting that other identified suppressors may also reside in the Ras pathway. The RAS screen benefited from information on known pathway members that could be tested in secondary screens. For example, KSR mutations also interacted with RAF, MAPK mutations, and a dominant-negative RAS mutation (Therrien et al., 1995). However, in early studies on unexplored novel genes, these secondary screens may be a luxury. The *Drosophila* eye has become the most common site of genetic screens, since the modifier phenotype is easy to score (smooth to rough eye structure), eye function is dispensable to the viability of a laboratory fly, and ~70% of

all *Drosophila* genes are expressed in the developing eye (Miklos & Rubin, 1996).

In a similar spirit to yeast multicopy plasmid suppression screens, a P-element-based GOF screen was developed to by-pass the robustness problems of P-element insertions as a knockout strategy (Rorth, 1996). In yeast, multicopy suppressor screens have demonstrated that overexpression can identify biologically relevant genetic interactions. The central premise is that, if mis/overexpression of one gene suppresses the phenotype of another gene, their products are likely to be directly involved in the same process. This dominant overexpression screen in *Drosophila* employs insertional mutagenesis with a P-element that contains GAL4-inducible promoter (UAS sites). Since P-elements tend to insert close to a start of transcription, a cell that expresses GAL4 initiates transcripts in the gene adjacent to the UAS site. Thus, one can design a screen to identify genes that under high levels of transcription modify a given phenotype and the screen can be restricted to a cell type by using specific promoters expressing GAL4 (Rorth, 1996). Also, 2300 independent insertions (EP lines) have been mapped and are available as a public resource for genetic screens. These lines have been used successfully as a screening strategy to identify components of biological processes, including wing and eye development and neuromuscular and cell death phenotypes (Hay et al., 1997; Rorth et al., 1998; Kraut et al., 2001).

Overexpression modifier screens can minimize the problem of genetic redundancy where LOF mutations would not be revealed. Overexpression modifier mutations can also complement information from LOF mutations as well as reveal gene functions not discovered in LOF mutations. For example, to find genes that alters Ras signaling, Huang and Rubin screened a Ras pathway-dependent rough eye phenotype for EP lines that could modify the phenotype. This approach identified several Ras pathway molecules discovered in the dominant modifier LOF screen described above as well as novel genes (Hay et al., 1997; Huang & Rubin, 2000). The risk in these screens is that high and inappropriate expression of a protein can perturb a system and still not be relevant (e.g., if misexpression causes a rougher eye due to cell lethality, leading to a gene identified as a “modifier” from nonspecific effects).

#### 5.4.6. Clonal screens

In addition to modifier screens, an elegant use of the FRT/FLP system to screen mutant chromosomes can be employed routinely. In this technique, mutant clones induced by mitotic recombination are screened for phenotypes where the FLP enzyme is induced on mutagenized (EMS or P-element) chromosomes containing FRT sites. This was first shown by Xu and Rubin (1993) to identify loci that affect growth in development on the basis of a visible adult tumor outgrowth mutant phenotype. They identified the tumor suppressor warts (a.k.a. lats kinase) and went on to show that LATS1-deficient mice sponta-

neously develop tumors (St John et al., 1999). FRT/FLP screens can also be combined with the GAL4/UAS system to target the expression of FLP in UAS-FLP lines for very directed mosaic screens (Duffy et al., 1998). St Johnston (2002) reviewed the many different uses and ingenious tricks that have been developed to allow exquisite control of genetic screening, leading to mutant detection and reduced false positives.

### 5.5. Post-genomic fly world

In the previous *Drosophila* sections, we have reviewed established techniques and genome information, and the following sections will review emerging genomics technologies that are being applied in combination with *Drosophila* genetics and sequence information.

#### 5.5.1. Cell-based “genetic” screens

In addition to RNAi as a “knockout” tool to study gene function in animals, *Drosophila* cell-based systems are extremely amenable to RNAi. dsRNA added to *Drosophila* cell lines (even without transfection!) is 95% effective in ablation of protein expression (Caplen et al., 2000; Clemens et al., 2000). For example, the insulin signaling pathway was studied for the efficiency of RNAi in *Drosophila* S2 cells (Caplen et al., 2000; Clemens et al., 2000). As expected from knowledge of the insulin pathway, inhibiting the expression of MAPKK by dsRNA prevents insulin-stimulated phosphorylation of MAPK. In another branch of the insulin pathway, dsRNA directed against PTEN, a negative regulator of insulin signaling, leads to constitutive activation of the insulin-responsive PI3K pathway. In a similar experiment on Ras pathway dissection, proteins such as KSR, which were discovered earlier in Ras genetic screens as acting downstream of Ras activation, were tested by RNAi for contributions to MAPK activation (Anselmo et al., 2002). Therefore, RNAi, combined with established biochemical reagents, allows deeper characterization of complex signaling pathways.

RNAi technologies for use in mammalian cell-based systems are rapidly evolving; however, the ease, cost, efficiency, and reproducibility using RNAi in S2 cells will allow for routine genome-wide functional analysis (Elbashir et al., 2001; Tuschl, 2002). For example, RNAi was used to identify a cellular tyrosine kinase that acts upstream of the phosphorylation of Dscam, a protein found to be important in axonal pathfinding (Muda et al., 2002). Of 6 RNAi reagents directed to suspected kinases, the RNAi Src42A was found to be able to decrease tyrosine phosphorylation on Dscam in S2 cells. This could be scaled up easily in S2 cells to test all 200+ *Drosophila* kinases for changes in a phosphorylation event. In *Drosophila*, RNAi is most effectively induced by dsRNA of over ~80 nucleotides (nt) in length, which are easy to PCR generate (Clemens et al., 2000). Conversely, in mammalian cells, an RNAi-like inhibition of gene expression is mediated by 21- to 23-nt

oligos, which are expensive to purchase, although oligo vectors for RNAi are being developed. Presently, the expense of RNA oligos makes mammalian cell-based “genetic” screens prohibitive. Also, the smaller, less redundant genome of *Drosophila* may be more revealing. Notwithstanding, mammalian RNAi will be an important resource for rapid validation of *Drosophila* targets in vertebrate biology.

#### 5.5.2. Transcriptional profiling

Microarrays are an invaluable tool for comparing the RNA expression profiles of different samples. Transcript abundance and regulation is one measure of gene function, and coregulated genes in complex multiorganisms suggest involvement in the same biological process. However, genome-wide expression analysis tends to result in a vast number of genes that are differentially regulated. In *Drosophila*, experiments can be designed to compare samples that are carefully matched in terms of developmental stage and genetic background, which allows identification of true biological changes in gene expression. Substantive clues are gained by monitoring levels of gene expression in normal and mutant conditions. Identification of most transcriptional units in *Drosophila* allows construction of microarrays, with a set of genes representing nearly every encoded protein. In *Drosophila*, DNA microarrays have been used to monitor the global transcriptional status and include the 2 most common microarray technologies: oligonucleotides affixed to a surface of a silicon chip (Affymetrix FlyChips) or as PCR products spotted onto glass slides (White et al., 1999).

*Drosophila* microarrays have identified the genetic signature of biological processes, such as metamorphosis, oscillating gene expression in circadian cycles, and immune response, and these studies have shown the value of whole-genome transcriptional profiling to facilitate understanding of tissue- and cell-type gene expression (White et al., 1999; McDonald & Rosbash, 2001; De Gregorio et al., 2002). In a bold series of experiments, global gene dissection with microarrays attacked the broad process and often intractable biology of aging (Pletcher et al., 2002). Aging physiology is a problematic experimental field because it is not one function but an endogenous progressive deterioration of fitness that involves many tissues and biological processes. Pletcher et al. compared age-dependent profiles from animals under standard diet conditions as well as calorically restricted diet, a condition well known to extend lifespan in many experimental models. The strength of the study comes from the stringent statistical criteria with replicate arrays using 3 independent *Drosophila* samples at 8 time points. Approximately 7% of the 14,000 fly genes changed expression. To ascertain biological meaning in this large list of genes, they organized the regulated genes according to the Gene Ontology annotation database (<http://www.geneontology.org>). As discussed in Section 5.3, GO annotates genes according to known structure, biological process, and molecular function.

Genes in the same functional category whose expression profile changes with age or diet are of potential interest in aging research. Known genes involved in aging such as stress response genes were clustered together. Also, clustered with these genes were other categories of genes not previously associated with aging, such as the immune response genes. It will be unlikely that all genes that consistently changed with time are a cause or effect of the aging process, but this study abounds with new candidate genes and concepts that can be investigated. Once confirmed in genetic mutant analysis, these results can be tested in mammalian systems.

In a more defined study, Furlong et al. (2001) presented a powerful demonstration of transcriptional profiling to comprehensively identify genes involved in muscle development in *Drosophila*. In *Drosophila*, *twist*, a helix-loop-helix transcription factor, is an essential player in mesoderm formation. *Twist* LOF mutants prevent mesoderm development; conversely, ubiquitous *twist* converts cells destined to become neurons to a muscle fate. They compared gene profiles in *Drosophila* stage-matched embryos of wild-type and *twist* mutants to identify genes that are down-regulated in *twist* mutants but up-regulated in response to ubiquitous *twist* expression. Of the 5000 genes on the microarray, 51 had significant expression level changes in *twist* mutants, including genes known to be regulated by *twist*. Self-organizing maps were used to cluster genes according to similar expression patterns and to identify coregulated genes. In situ hybridizations were undertaken to confirm that these genes were expressed in mesoderm at the correct time. *Gleeful* (*gfl*), a novel *Drosophila* homolog of the vertebrate Gli proteins, appeared in transcriptional profiles as associated with muscle development: high in ectopic expression and low in LOF phenotypes. Furthermore, embryo injections of *gfl* RNAi caused severe loss and total disruption of somatic muscle. Conversely, *gfl* was able to induce muscle gene expression when expressed with UAS-*gfl* in tissues normally destined to other cell fates. This example demonstrates that combining RNAi, ectopic expression, and defined mutations with transcriptional profiling can discover new protein networks.

#### 5.5.3. Animal sorting

In addition to the discovery of *gfl*, Furlong et al. (2001) introduced an automated animal sorting machine to collect large quantities of mutant embryo. In this case, the sorter separates embryos homozygous for the *twist* mutations from their heterozygous siblings based on GFP expression. More than 99% of the animals sorted as non-GFP had the *twist* phenotype. The embryo sorter is much faster than a manual method. Therefore, a collection of large numbers of embryos during 3 consecutive narrow time points allows association of differentially expressed genes with different phases of mesoderm development. *Drosophila* embryo and larvae automated sorting will allow large quantities of genetically defined mRNA for microarray analysis or biochemistry. Also, it is now possible to design genetic screens

to identify mutant embryos or larvae in an animal sorter by the presence or absence of a marker.

#### 5.5.4. Chemical genetics

Chemical genetics as defined by using small molecules as probes of biological function is an emerging discipline that can be applied to *Drosophila* (Chan, 2002). In *Drosophila*, this can be applied to designing screens based on the concept that, if a mutation in one gene makes animals resistant to chemically induced phenotype, the chemical is likely to target the same biological network as wild-type version of the mutant gene product.

In early experiments on insecticide resistance, a chloride ion channel gene was found to be mutant in *Drosophila* populations resistant to the insecticide cyclodiene. Further studies demonstrated this ion channel as the direct target of cyclodiene (French-Constant et al., 1993). Today, there exist many examples of mutations in *Drosophila* orthologs of mammalian genes that are responsive to pharmacological agents. For example, RAS farnesyl protein transferase inhibitors selectively suppress the activated Ras alleles (Kauffmann et al., 1995). In another human disease relevant example, fly *bubblegum* mutants accumulate very long fatty acids (VLCFA), leading to neurodegeneration. Similarly, patients with adrenoleukodystrophy display progressive neurodegeneration due to accumulation of VLCFA. “Lorenzo’s oil,” which is used clinically to maintain low VLCFA levels, can also rescue the mutant phenotype when fed to *bubblegum* flies (Min & Benzer, 1999).

Mechanism of a compound’s action can also be explored with genomic technologies such as DNA microarrays. For example, feeding drugs to flies and testing global gene changes at discrete time points would be informative. In line with the aging studies described above, other groups have combined drug treatment of aging flies with transcriptional profiling (Zou et al., 2000; Kang et al., 2002). Mutations in worms, flies, and mice that increase lifespan also increase resistance to oxidative stress; however, the aging correlation to oxidative stress is poorly understood. Zou et al. (2000) evaluated the relationship between aging and oxidative stress response by comparing age-dependent transcript level changes with those caused by oxidative stress induced by the free radical-generating compound paraquat. In another study, 4-phenylbutyrate (PBA), a histone deacetylase, when fed to *Drosophila*, was shown to increase lifespan and to increase global histone acetylation (Kang et al., 2002). Transcriptional profiling with paraquat or PBA in aging flies compared with no drug treatment demonstrated coregulation of genes involved in metabolism, stress response, and detoxification. The difficulty in comparing the studies is the lack of standard annotation such as Gene Ontology. Deciphering the meaning of whole-genome gene expression is in its infancy, and careful and consistent standards needed to be applied across the studies. However, it is intriguing to see biological processes dissected in a way that reveals new views for future exploration.

The conservation of pharmacology combined with the genetically tractable tools available in *Drosophila* will allow identification of the pathway of drug action. Transcript profiles can provide a “snapshot” of molecular behavior but also may identify genes critical to drug action. For example, RNAi or mutation in a gene involved in the drug action may perturb the “snapshot.” Also, the possibility exists to screen for compounds that have a specific phenotype or profile in *Drosophila*. For example, a few validated candidate genes identified by transcriptional profiling as modulated by aging (or any process) can be markers for quantitative PCR in screens for lead compounds.

In conclusion, *Drosophila* research offers a mammalian relevant system where orthologous proteins can be studied by mutations and other genetic tricks to modify functional pathways to reveal their function. Once hooked into a pathway, many new genomics tools can be brought to bear on a problem. Advances in *Drosophila* research allow the combination of genome sequence information, genome-wide cDNA, mapping protein interactions, gene expression profile, and genome-wide mutations in an unprecedented dissection of a complex organism.

While *Drosophila* models of human disease are powerful, and the genetic tools available in *Drosophila* are somewhat unique, much of drug discovery depends on rodent models. The mouse genome has been completed recently, and the technology for genetically manipulating mice is ever evolving.

## 6. Mouse models

Historically, drug discovery has consisted of a linear process whereby a large number of compounds are tested in relevant models. It is estimated that currently only 483 targets account for all drugs now on the market (Drews, 2000; Reiss, 2001). The publication of human sequences has resulted in the availability of large amounts of data that are potentially directly relevant to the discovery of novel and useful drug targets, which will necessitate a change in this linear process. Of the ~30,000 genes identified in the human genome, estimates are that 3000–10,000 might be potentially interesting drug targets (Drews, 1996). This places the emphasis of drug discovery on the elucidation of unknown gene function in contrast to the common screening of known targets. The elucidation of novel gene function, however, is not a simple matter. While human genetic studies have been useful in identifying genes implicated in disease, the cloning of human disease genes is often ponderous and expensive. It is now possible to quickly determine the function of a gene-utilizing genomics and gene knockout technology in the mouse. Testing in animals is an indispensable part of drug discovery and development, where the purpose is to validate a potential target for intervention and to assess the liabilities associated with a drug candidate. Mice are widely utilized in drug discovery,

for basic pharmacokinetic studies, for proof of concept behavioral studies, and for toxicology. Disease models in rodents remain at the heart of animal testing programs, while toxicology studies based on animal responses are fundamental to the safety and success of a compound of interest. Genetic approaches have already influenced drug discovery, and the field of genomics promises to continue to broaden the impact of such animal models in the future.

### 6.1. Recent and current uses

Traditionally, drug discovery has operated either by the screening of large numbers of chemical and natural structures for potential therapeutic efficacy or by a hypothesis-driven approach. Using this latter method, a target of interest is identified from clinical evidence or published literature and then developed. Drug targets are tested against a library of molecules to identify those that interact with high affinity. These high-affinity molecules are referred to as hits and are screened to identify lead compounds. Lead compounds are then tested in experimental animals, including rats and mice, with special attention being paid to the behavior of those compounds in animal models of human disease. There are also extensive tests of safety and tolerability in animals that compounds are required to pass through before entering clinical trials in humans. While the traditional drug discovery process has yielded novel and important disease-mitigating therapies, it is clear that the process improvement is needed. Many drugs continue to fail in clinical trials due to a lack of efficacy or toxicity. In many cases, it is clear that the proper degree of target validation has been lacking. The ability to create mouse models lacking any gene will clearly have an impact on the quality of drug targets taken forward into clinical trials in the future. In fact, within several years, there will be no technological reason not to have a mouse model lacking any gene of interest.

### 6.2. Hypothesis-driven animal models

#### 6.2.1. Transgenics and knockouts

One of the most extensive utilities of mice in drug discovery is for models of human disease. The genome of the mouse is strikingly similar to that of humans, and ~95% of the genes are highly conserved. In addition, the mouse has the added benefits of easy manipulation and cost efficiency. Transgenic and knockout technology allows researchers to add or subtract genes from the mouse genome, thereby implicating those genes in human disease processes. This technology has become industrialized, and it is expected that most mouse genes will have been knocked out in the next 10 years. Mouse models are usually created through forward or reverse genetics. In reverse genetics, researchers begin with the knowledge that a given human gene is likely involved in the human disease pathway. Mouse models that lack the particular gene, overexpress the gene, or contain mutant forms of the gene are created in order to understand the



normal role of the gene and to phenocopy the human disease in mice. An example of this type of an approach is the corticosteroid receptor. Corticosteroid receptors have been hypothesized to be involved in the onset of depression-like illness and have led to a focus on brain neuropeptide receptors. Specifically, the corticotropin-releasing hormone (CRH) receptor has been considered a candidate drug target in the development of antidepressant therapy. Clinical evidence suggests a role for CRH in depression as the hormonal response of cortisol is shifted to higher values in depressed patients, indicating a lower negative feedback capacity of CRH receptors. Furthermore, postmortem examination of depressed patients shows increased numbers of CRH-expressing neurons. Injection of CRH into the brains of mice induces a number of behavioral effects that are similar to depressive symptoms seen in humans. Mouse models lacking the CRH genes support results from the clinical population and have indicated that CRH antagonists might be a viable approach for antidepressant therapy. Two distinct CRH receptor subtypes (CRH-R1 and CRH-R2) exist, and it was unclear which of these proteins should be modulated to give an antidepressant effect. Mice were created specifically lacking the gene coding for either CRH-R1 or CRH-R2 in order to answer this question. CRH-R1 mice exhibit decreased anxiety (Timpl et al., 1998), while CRH-R2 mice either show no behavioral change or demonstrate an increased anxiety-like response. These findings suggested that the CRH-R1 and not CRH-R2 should be targeted to develop anxiolytics and antidepressants (Bale et al., 2000; Kishimoto et al., 2000). Whereas previous compounds may have been developed against the entire family of CRH-R genes, the mouse knockout findings suggest that a CRH-R1-specific compound would likely have the best efficacy with least amount of potential for toxicity.

### 6.2.2. Toxicology and lead profiling

In addition to being utilized to find the functions of genes, mouse models can also aid in the field of risk assessment for novel therapies. The proper assessment of drug metabolism and toxicity are crucial to drug discovery, and the ability of mouse models to aid in this stage is tremendous. Toxicities related to drug-metabolizing enzyme systems, drug-induced immunotoxicity, genotoxicity, and carcinogenicity are all aspects of risk assessment. Mice with alterations in enzymes involved in metabolism (CYP450 enzyme transgenics/knockouts or glutathione and glucuronyl transferase transgenics/knockouts) aid in understanding drug metabolism and toxicity (Nebert & Duffy, 1997; Rudmann & Durham, 1999). As an example, Liang et al. (1996) have created mice lacking the gene coding for the cytochrome P450 1A2 (CYP1A2) enzyme. This enzyme is a predominantly hepatic phase I drug-metabolizing enzyme known to be important in the metabolism of a number of chemicals. Mice lacking a functional CYP1A2 enzyme are viable and exhibit normal development and provide a useful resource for investigators wishing to understand the role of

the CYP1A2 enzyme in the metabolism of their compounds (Liang et al., 1996).

### 6.2.3. Carcinogenesis

Another area of risk assessment where the combination of mice and genetics have been useful is to test compounds for carcinogenicity. A typical model used in determining whether a compound is carcinogenic is a 2-year rodent bioassay (Tennant et al., 1995). This time period is an eternity in drug discovery (and in the lives of cancer patients awaiting novel therapies). The challenge is to increase productivity and decrease time lines of carcinogenicity tests without sacrificing the quality and safety of the resulting compounds. Transgenic mice can offer short-term models to identify potential risk. Mice have been created that carry genes which are mutagenic (Mirsalis et al., 1994; Morrison & Ashby, 1994) or contain inactivated tumor suppressor genes, which can contribute to carcinogenesis (Stewart et al., 1984; Donehower et al., 1992; Tennant et al., 1993). One of the most frequent molecular changes in a variety of human cancers is mutation of the p53 tumor suppressor gene, which is critical to cell cycle control and DNA repair (Hartwell, 1992; Kastan et al., 1992; Zambetti & Levine, 1993). The p53<sup>+/-</sup> hemizygous knockout mouse model is phenotypically stable and develops tumors during the relatively short 6-month study period in response to chemical and physical stimuli. The results from this mouse line show a high concordance with genotoxic rodent carcinogens (Tennant et al., 1993, 1995) yet take less than one third of the time to complete. The p53 knockout mouse is one of several genetically altered mice whose use may increase the sensitivity and decrease the time and cost of risk assessment bioassays (Sands et al., 1994; Tennant et al., 1995). As with any experimental model system, data obtained from genetically altered mice must be interpreted carefully. The complete inactivation of a gene may result in altered expression of related genes or physiologic compensation for the loss of the gene product. Consideration must also be given to the genetic background of the mouse strain and the impact of strain variability on disease or toxicity models. Despite these potential limitations, knockout mice provide a powerful tool for the advancement of drugs in the pharmaceutical industry.

## 6.3. The post-genomic world

### 6.3.1. Genomic tools

Genes encoding traditional drug targets are only a small fraction of those recently discovered. In the era of functional genomics, hypothesis-driven research will be complemented by a new strategy that begins with a gene and endeavors to quickly link the gene to biological processes. As discussed earlier in this review, this type of approach, when combined with powerful genomics tools, has proved quite successful in invertebrate models. Many of the same genetic and genomics tools utilized in invertebrate systems are now



available in mouse and other mammalian models. These techniques range from DNA microarrays to expression mapping, database mining, and forward and reverse genetics. Much of drug discovery in the post-genomics era begins with bioinformatic identification of a gene sequence (Dollery, 1999). Identification of this sequence leads to estimates of expression patterns (Roos, 2001). Tissue distribution of the potential target can be estimated by electronic scanning of databases of sequences derived from tissue-specific cDNA libraries. A potential relationship to disease states is inferred by information stored in libraries that compare tissue from healthy and diseased individuals, and further insight into function can be enhanced at a later stage by testing transgenic or knockout animals. In addition, tools, such as antisense technology or herpes viral vectors, might take an increased importance in elucidating/gathering proof of concept for these novel genomic targets.

### 6.3.2. *Chips*

Given the large market for neuropsychiatric diseases, evaluation of novel targets for neuroscience diseases has the potential for meeting one of the largest unmet medical needs. One approach to dealing with these poorly understood disorders is microarray systems. Microarray systems have been adopted for analysis of human and animal models to aid target selection and validation. Oligonucleotide or cDNA chips permit comparisons of gene expression in diseased versus normal tissue, treated versus untreated individuals, and responders versus nonresponders in humans. Changes in gene expression in these cases allows for a better understanding of the global effects of a given drug on different mouse tissues and can be utilized in conjunction with laser capture to examine regional gene expression in brain areas hypothesized to underlie a disease (Dent et al., 2001). While the use of microarrays in the field of neuroscience is in its infancy, the technique holds promise for the future. It is not clear yet that gene expression changes caused by an agent *in vitro* will reflect those produced *in vivo*. In order to address these questions, microarray technology has been utilized to characterize several known toxins *in vivo* by observing gene expression changes (Waring et al., 2001). Results demonstrated that gene expression profiles for compounds with similar toxic mechanisms showed similar profiles but did not completely overlap, indicating that each compound was unique. That such marked similarities were found among compounds, however, suggests that large-scale analysis of gene expression using microarray technology has promise in correlating gene expression patterns *in vivo* as diagnostic tools for toxicology and pathology. Whether microarray technology eventually leads to approved diagnostic tests remains to be determined.

### 6.3.3. *Industrial reverse and forward genetics*

The release of the human and mouse genome maps combined with novel genomics tools will certainly further influence drug discovery and the utility mouse models. As a

more direct alternative to screening multiple compounds and validating targets based on compound activity, the deletion or overexpression of candidate genes in animals has proven to be a useful approach. In the past, however, the process for making a knockout mouse has been arduous and expensive. In addition, it was often difficult to identify and purify the mouse homolog of a given human disease gene. The complete mouse genome sequence has enabled the design of specific PCR primers for any given mouse gene and allowed for most genes to be cloned into gene targeting vectors for the creation of knockout mice. The sequencing of the genomes of multiple mouse strains has allowed polymorphisms among mouse strains to be identified and utilized for more rapid gene mapping by forward genetics approaches. It is only in the last few years that the both forward and reverse genetics in the mouse have been attempted on a large industrial scale. This large-scale usage of mice requires a substantial investment of time and an expensive infrastructure. One example of a company that has industrialized mouse reverse genetics is Lexicon Pharmaceuticals. Lexicon utilizes gene knockout technology to define the functions of genes for the discovery of pharmaceutical products in cancer, cardiovascular disease, immune disorders, neurological disease, diabetes, and obesity. They knock genes out in the mouse utilizing homologous recombination or gene-trapping technologies. These technologies target specific genes for knockout in mouse embryonic stem (ES) cells and then use these cells to create mouse strains lacking any given gene. Many of these genes are selected based on biological hypotheses. However, the industrial scale of the project and the speed of the technology allows for one to choose large numbers of genes of unknown function. Complete analysis of the effects the missing gene has on the health of the animal allows inferences to be drawn about the function and importance of the missing gene in disease processes. The ability to rapidly assess the *in vivo* function of many mammalian genes and to associate defects in those genes with disease processes is very powerful. While this is outside the scope of the traditional pharmaceutical approach, it clearly complements the efforts of pharmaceutical companies and is a way to assess the *in vivo* potential of any given gene product as a novel drug target.

As opposed to the reverse genetics approach of knocking out predefined genes, another approach to shifting through the large numbers of unknown targets is to randomly mutagenize the entire mouse genome, screen for mice that have disease symptoms, and then work your way back to the disease-causing gene. The ENU Mutagenesis Program (<http://www.mgu.har.mrc.ac.uk/mutabase/>) is a large-scale mouse genetics effort designed to identify new phenotypes of relevance to human disease. A collaboration between the Medical Research Council in Harwell and Glaxo-SmithKline has generated large numbers of new mouse phenotypes. The mutagenesis screen identified ~500 novel mouse phenotypes classified into the following disease/disorder categories: diabetes, neurological/neurobehavioral, and bone

disorders. Such companies as Ingenium Pharmaceuticals have also undertaken other large-scale projects in forward genetics. The rate-limiting step in this process is the ability to rapidly clone out the disease-causing genes. However, this limitation seems to be receding with advances in mapping techniques. One potential drawback to this type of analysis is that once the gene responsible for the underlying mouse phenotype is identified, it may fall outside of the gene families and proteins whose functions are understood and to which medicinal chemists are able to target small molecules. However, if we do not understand the normal function of a gene, it can take a long time to put it in its proper biological context. For instance, the gene that is causative for cystic fibrosis was discovered many years ago, yet a lack of understanding of the normal function of the gene has resulted in a delay in the development of significant therapies.

#### 6.4. Complexities

It is clear that a phenotypic change rendered in isolated mammalian cells by a compound is one level of validation that a target may have a therapeutic effect. The effects of that same compound on lower model organisms, such as worms and flies, provides a second level of validation. In most cases, however, drug target credibility is not reached until phenotypic changes can be reproducibly induced in a mammalian animal model.

One important caveat to simple animal models is in studying polygenic diseases. We know, for instance, that many neurological and psychiatric diseases have multigenic polygenic traits (i.e., each individual gene exerts only a small effect on risk). Thus, a gene-by-gene analysis may not give a complete picture of how genes act together to mediate these complex diseases. Furthermore, genomics approaches may not be sufficient to integrate a genetic understanding of the functioning of the intact organism with environmental variables. Understanding how genes function individually, however, is the building block to understanding the more complex mechanisms by which they interact. Mouse, rat, and other mammalian species continue to be utilized as important models of human diseases and play a vital role in the discoveries and safety of new medicines. Genomics tools have evolved and are helping to reveal the complexities of biology and human disease. Indeed, they are paving the way towards the next generation of novel, safer, and more effective medicines.

#### 7. Conclusions and perspectives

The use of animal models in drug discovery is an essential component of the entire drug discovery process. Genetic models allow investigators to probe complex biological questions that would take many years to answer and in some cases would require experiments that are considered unethical in humans. Moving from the unicellular systems

such as yeast to the relatively more cumbersome rodent models, one exchanges speed for relevance to human disease. Experiments done in yeast are often lightning fast in comparison with similar experiments designed to answer the same question in mouse models. The results in the mouse models, however, more often reflect the disease state and biology in humans. It is clear that only the proper and intelligent use of a complement of animal models ensures that the process goes forward quickly and accurately. The continued development and use of these model systems and their tools will be at the forefront of the development of tomorrow's safe and efficacious disease-altering molecules for many years to come.

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## Review

## Toll-like receptors; their physiological role and signal transduction system

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Received 18 September 2000; accepted 12 December 2000

## Abstract

*Drosophila* Toll protein is a transmembrane receptor whose function is to recognize the invasion of microorganisms as well as to establish dorso-ventral polarity. Recently, mammalian homologues of Toll, designated as Toll-like receptors (TLRs) have been discovered. So far, six members (TLR1-6) have been reported and two of these, TLR2 and TLR4, have been shown to be essential for the recognition of distinct bacterial cell wall components. TLR2 discriminates peptidoglycan (PGN), lipoprotein, lipoarabinomannan (LAM) and zymosan, whereas TLR4 recognizes lipopolysaccharide (LPS), lipoteichoic acid (LTA) and Taxol. Bacterial components elicit the activation of an intracellular signaling cascade via TLR in a similar way to that occurs upon ligand binding to IL-1 receptor (IL-1R). This signaling pathway leads to the activation of a transcription factor NF- $\kappa$ B and c-Jun N-terminal kinase (JNK), which initiate the transcription of proinflammatory cytokine genes. Particularly, analysis of knockout mice revealed a pivotal role for MyD88 in the signaling of the TLR/IL-1R family. Taken together, TLRs and the downstream signaling pathway play a key role in innate immune recognition and in subsequent activation of adaptive immunity. © 2001 Published by Elsevier Science B.V.

**Keywords:** Toll-like receptor; MyD88; NF- $\kappa$ B; Macrophages; Knockout mice; Inflammatory cytokine

## 1. Introduction

Invading microbes are killed by two different classes of immune system, innate and adaptive. The innate immune system, which is responsible for the initial and rapid host defense, mediates inflammatory

responses and subsequently instructs adaptive immunity that takes several weeks to generate. The hallmark of the innate immune system is the usage of germline-encoded pattern recognition receptors to sense the invading pathogens [1]. Although microorganisms have tremendous variety, they possess common structural features, for example lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN), lipoteichoic acid (LTA) from Gram-positive bacteria, lipoarabinomannan (LAM) from mycobacterium, unmethylated DNA and bacterial lipoproteins. The pattern recognition receptors

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sense the presence of these bacterial components and activate common equipment to mediate responses for the elimination of invading microorganisms. This evolutionary ancient bacterial recognition system is found even in the *Drosophila* immune response, so called Toll signaling pathway [2,3]. Recent studies revealed that mammalian TLRs, homologues of *Drosophila* Toll, are key molecules for recognizing bacterial components to evoke inflammatory response. Here, we will review recent advances in the research of the bacterial recognition system via TLRs and their signaling pathway.

## 2. The host defense in *Drosophila*

A unique and important part of the *Drosophila* immune response is the synthesis of antimicrobial peptides against microbial infection [4]. The Toll signaling pathway has been shown to regulate the rapid production of appropriate antimicrobial peptides in response to microorganisms. Toll was originally identified as an essential component of the pathway that determines the dorsal–ventral axis in early *Drosophila* embryogenesis [5]. Toll is a type I transmembrane receptor whose extracellular region contains leucine-rich repeat (LRR) motifs. The cytoplasmic domain of Toll possesses similarity with that of the mammalian Interleukin-1 receptor (IL-1R) family, designated as Toll/IL-1R (TIR) homology domain [3]. Toll controls the expression of the anti-fungal peptide drosomycin. Toll-mutant flies are susceptible to fungal infection, while the expression of antibacterial peptides is not affected [2]. Genetic experiments in *Drosophila* have identified signaling molecules required for the Toll pathway, an adaptor protein Tube and serine/threonine kinase Pelle [6]. Both Tube- and Pelle-mutant flies showed reduced drosomycin induction after immune challenge [2]. Subsequent to activation of Pelle, phosphorylation and degradation of Cactus couples with the nuclear translocation of a Rel-type transcription factor Dif, to induce the transcription of the drosomycin gene [7]. This Pelle/Cactus/Dif signaling cassette is homologous to a mammalian IL-1R signaling module, IRAK/I $\kappa$ B/NF- $\kappa$ B, respectively.

In *Drosophila*, the Toll family consists of eight members [8]. Among them, 18-wheeler has been

reported to control the synthesis of an antibacterial peptide, attacin [9]. It was shown that anti-fungal peptide production was not affected in 18-wheeler mutant flies. Thus, *Drosophila* distinguishes the different types of invading microorganisms by different Toll-signaling pathways. Activation of different signaling pathways results in the production of anti-microbial peptides appropriate to the particular type of pathogen [10].

## 3. Toll-like receptor family

In 1997, human homologues of Toll, designated as Toll-like receptors (TLRs), were discovered [11,12]. The TLR family has six published members and is now expanding up to 12 members (our unpublished data) [13,14]. Mammalian TLR, as well as *Drosophila* Toll, is characterized by extracellular LRR motifs and a cytoplasmic TIR homology domain (Fig. 1). LRRs are short protein modules of 20 to 29 amino acids found in a diverse group of proteins, including CD14, platelet glycoprotein 1b and RP105. RP105 is a transmembrane protein that consists of an extracellular LRR domain and a short cytoplasmic tail [15]. RP105 is expressed on B lymphocytes and on dendritic cells, associating with a molecule MD-1 [16]. Cross-linking of RP105 resulted in B cell proliferation and augmentation of co-stimulatory molecule expression. Recently, Ogata et al. [17] reported that targeted disruption of RP105 resulted in the impaired proliferative response of B cells to LPS. They speculated that RP105-MD-1 pair was a component of the LPS receptor complex in B cells. Plant disease-resistance proteins from tobacco, thale cress and flax also have these protein modules. The recently identified *Arabidopsis* FLS2 protein is a transmembrane receptor, which features extracellular LRRs and a cytoplasmic serine/threonine kinase domain. FLS2 has been shown to be involved in the perception of flagellin, the main protein of bacterial flagella, by *Arabidopsis*. Flagellin treatment caused growth inhibition in wild-type plants, while it did not induce any response in the plants in which the *FLS2* locus was mutated [18]. The presence of LRR modules in human, fruit fly and plant proteins suggests that LRR proteins take part in evolutionary primitive forms of host defense.

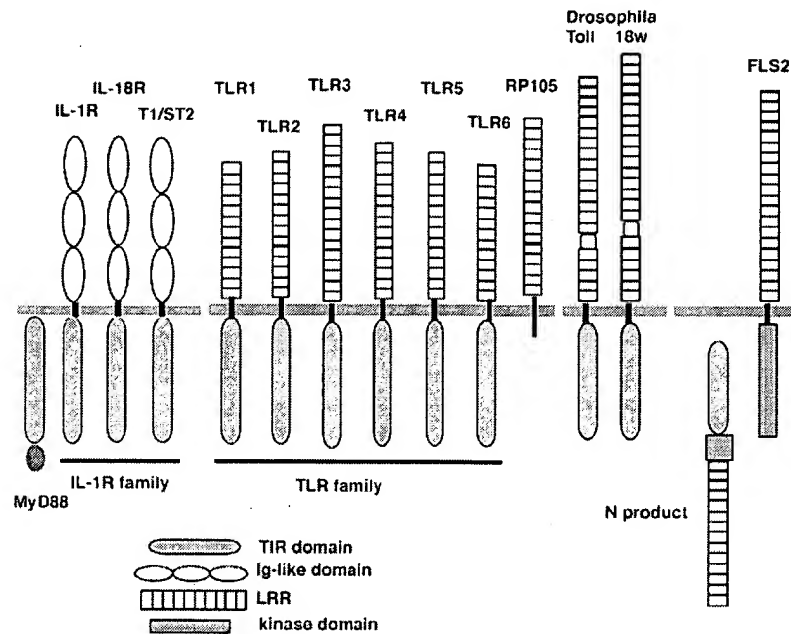


Fig. 1. The IL-1R/TLR superfamily. The receptor proteins, which possess LRR and/or TIR homology domain, participate in innate immune responses. TIR homology domain is common to TLRs, IL-1R family members such as IL-1R, IL-18R and T1/ST2, the intracellular protein MyD88 and plant tobacco disease resistance gene product N product. Ig-like modules in the IL-1R family distinguish it from TLR. In addition to TLRs, RP105, plant N product and FLS2 feature LRR motifs. MyD88 has a death domain and the cytoplasmic portion of FLS2 features serine/threonine kinase.

The cytoplasmic TIR homology domain of the TLR family is similar to that of IL-1R family proteins, such as IL-1R, IL-18R and T1/ST2 [19]. The extracellular immunoglobulin domain of IL-1R family distinguishes them from TLRs. The TIR homology domain is also found in an adaptor protein, MyD88, which interacts with that of IL-1R/TLR family members. The IL-1R/TLR family is thought to share a common signaling pathway leading to the activation of transcription factor NF- $\kappa$ B. Medzhitov et al. [11] showed that the expression of constitutive active TLR4 mutant results in NF- $\kappa$ B activation, surface expression of co-stimulatory molecules and cytokine production in THP-I cells.

The tissue distribution of TLRs has been studied extensively. A huge variety of cells express TLRs, such as cardiac myocytes, dermal endothelial cells, intestinal endothelial cells and  $\gamma\delta$  T cells [20–23]. These observations suggest that bacterial components directly activate various host cells for the initial host defense against microorganisms via TLRs.

It is of note that human TLR3 is selectively expressed in dendritic cells [24].

#### 4. Bacterial recognition via TLR family

Specific components common to a range of bacteria, so-called pathogen-associated molecular patterns (PAMPs), are potent activators of innate immune responses. These include LPS, PGN, LTA, LAM, lipoprotein and unmethylated DNA with CpG motif. PAMPs activate mammalian monocytes/macrophages and trigger the production of proinflammatory cytokines, such as Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-1, IL-6 and IL-12. They also elicit the production of inflammatory effector substances, for instance nitric oxide (NO) and eicosanoids. These effector molecules mediate inflammatory responses and drive T helper1 cell development, which leads to activation of adaptive immunity. Although these host



responses are important for eliminating infectious microorganisms, excess production of these cytokines can lead to septic shock and death.

The effects of LPS, a component of outer membrane of Gram-negative bacteria, have been studied extensively because of its property to activate host cells and to cause shock in animals. When LPS is present in the blood stream, it is immediately captured by LPS-binding protein (LBP), and then the LPS-LBP complex is transferred to CD14 located on surface of macrophages [25]. CD14 is known to bind LPS, and mice lacking CD14 are shown to be highly resistant to LPS-induced shock [26]. However, CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein without a transmembrane domain. Therefore, the existence of a bona fide signal transducing receptor had been predicted.

Initial reports demonstrating a relation between TLR and LPS recognition were published in 1998. Yang et al. [27] and Kirschning et al. [28] demonstrated that 293 human embryonic kidney cells transfected with human TLR2 showed LPS-induced NF- $\kappa$ B-dependent promoter activation. In spite of the initial characterization of TLR2 as a LPS receptor, subsequent studies did not support this conclusion. Poltorak et al. [29] and Qureshi et al. [30] described that the LPS hyporesponsiveness of C3H/HeJ and C57BL/10ScCr mouse strains is due to a mutation of the mouse *Tlr4* gene. A point mutation in the C3H/HeJ-derived *Tlr4* gene resulted in the replacement of proline with histidine in the cytoplasmic portion of TLR4 protein. Targeted disruption of the *Tlr4* gene by homologous recombination results in the abrogation of LPS responsiveness in macrophages and B cells [31]. Synthetic lipid A, which mimics an active principle of LPS, also failed to activate TLR4-deficient cells. In addition, TLR4-deficient mice are highly resistant to LPS-induced shock. Heine et al. [32] showed that Chinese hamster ovary (CHO) fibroblasts as well as Chinese hamsters, which have a truncated unfunctional TLR2, even display an ability to respond to LPS. However, they also described that expression of hamster full-length TLR2 conferred LPS responsiveness in 293 cells. To clarify whether TLR2 is responsible for the LPS recognition in vivo, we generated TLR2-deficient mice generated by gene targeting [33]. TLR2-deficient mice died on lethal challenge with LPS as well as wild-type mice.

Macrophages from TLR2-deficient mice produced amounts of cytokines comparable to the wild-type in response to LPS and synthetic lipid A. LPS-mediated B cell responses and activation of the signaling pathway in TLR2-deficient mice were also comparable to those of wild-type mice. Recently, it has been reported that a point mutation in human TLR4 also resulted in the LPS unresponsiveness [34]. More recently, Hirschfeld et al. [35] described that LPS repurified by phenol re-extraction protocol failed to activate TLR2-transfected cells, even though crude LPS could activate them. In contrast, TLR4-transfected cells showed NF- $\kappa$ B activation in response to LPS and synthetic lipid A. These observations indicate that neither human nor mouse TLR2 has the ability to confer LPS responsiveness, and that TLR2 may play a role in the recognition of LPS-contaminated endotoxin protein.

Some LPS, such as the lipid A analogues lipid A IVa and *Rhodobacter sphaeroides* lipid A, are known to exhibit a species-specific pharmacology. Both of them mimic LPS activity in hamster cells, but not in human cells. Lien et al. [36] showed that a difference of TLR4 from different species was responsible for this species-specific pharmacology, by expressing human and hamster TLR4 in CHO cells and THP-1 human cells. However, TLR4 requires other molecules to form a complex on the cell surface for efficient LPS signaling. Shimazu et al. [37] identified MD-2, a secreted protein that associates with the extracellular domain of TLR4. When MD-2 was co-expressed with TLR4 in Ba/F3 cells, it greatly enhanced LPS responsiveness. Taxol, an antitumor agent from a plant, is known to mimic the behavior of LPS in mice, but not in human. C3H/HeJ mice are known to lack the responsiveness to Taxol. Recently, it was reported that a difference of MD-2 between mouse and human determine the species-specificity [38]. Overexpression of mouse MD-2, but not human MD-2, with mouse or human TLR4 conferred Taxol responsiveness as assessed by NF- $\kappa$ B activation. We showed that TLR4-deficient mice lacked responses to LTA from Gram-positive bacteria, indicating that LTA is also recognized by TLR4 [33]. These reports show that TLR4 also plays a role in the recognition of not only LPS from Gram-negative bacteria, but also a component from Gram-positive bacteria.

In contrast to the role of TLR4 in LPS signaling, TLR2 is involved in the recognition of Gram-positive bacterial components. TLR2-deficient mice displayed an impaired production of TNF $\alpha$ , IL-6 and NO in response to several Gram-positive bacterial cell walls as well as *Staphylococcus aureus* PGN. In addition, *S. aureus* PGN-induced NF- $\kappa$ B DNA binding activity was also abrogated in TLR2-deficient macrophages, indicating that TLR2 is essential for the activation of the intracellular signaling pathway elicited by PGN. It was also reported that overexpression of TLR2 in 293 cells conferred responsiveness to *S. aureus*, *Bacillus subtilis* and PGN estimated by NF- $\kappa$ B activation [39]. Yoshimura et al. [40] demonstrated that CHO cells expressing TLR2, but not TLR4, could respond to *S. aureus* and *Streptococcus pneumoniae* by analyzing NF- $\kappa$ B DNA binding activity and surface CD25 expression. Overexpression of TLR2 in CHO cells also conferred the responsiveness to heat-killed *Listeria monocytogenes* and LAM from *Mycobacterium tuberculosis* [41,42]. Whole *M. avium* failed to activate CHO cells and macrophages from Chinese hamsters. In contrast, *M. tuberculosis* has been reported to activate cells via both TLR2 and TLR4 [43].

Underhill et al. [44] pointed out the correlation between macrophage phagocytosis and TLR-mediated inflammatory response. They demonstrated that TLR2 is recruited to the phagosomes of macrophages, after the treatment of cells with the yeast particle, zymosan. They also showed that RAW cells expressing mutated TLR2 showed abrogated inflammatory

responses to Gram-positive bacteria and zymosan. In contrast, overexpression of mutated TLR4 resulted in the inhibition of *S. minnesota*-induced cytokine production.

Bacterial lipoproteins are bacterial molecular patterns common in a wide spectrum of bacterial species. Brightbill et al. [45] and Aliprantis et al. [46] described that lipoproteins from *M. tuberculosis* and those from Gram-negative bacteria elicit inflammatory responses in TLR2-expressed 293 cells. 19-kDa *M. tuberculosis* lipoproteins induced IL-12 production from RAW cells via TLR2, and synthetic lipoprotein analogs from Gram-negative bacteria mediated apoptosis in THP-I cells through TLR2. Lipoproteins/lipopeptides from *Borrelia burgdorferi*, *Treponema pallidum* and *Mycoplasma fermentans* were also shown to stimulate cells through TLR2 [47,48]. In addition, TLR2-deficient mice lack TNF- $\alpha$ , IL-6 and nitric oxide production induced by the synthetic mycoplasmal lipopeptide MALP-2, in contrast to the normal responses of TLR4-deficient mice to MALP-2 [49]. MALP-2-induced NF- $\kappa$ B activation was abrogated in TLR2-deficient macrophages. These results indicate that the TLR2 protein commonly recognizes lipoproteins from most bacterial species.

Other than TLR2 and TLR4, TLR5 is implicated in anti-bacterial host defense [50]. The MOLF/Ei mouse strain is shown to be susceptible to infection with *Salmonella typhimurium*. One of the MOLF/Ei susceptibility loci was mapped to chromosome 1 where the mouse *Tlr5* gene mapped. It was observed

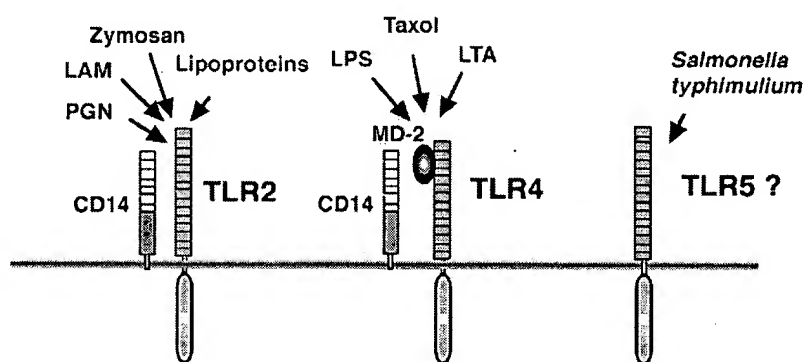


Fig. 2. Putative ligands for TLRs. TLR2 recognizes PGN, zymosan, LAM and lipoproteins from various bacteria. In contrast, TLR4 is required for the signaling of LPS from Gram-negative bacteria, LTA from Gram-positive bacteria and taxol. It is possible that TLR5 distinguishes a component of *Salmonella typhimurium*.

that the expression of TLR5 was reduced in MOLF/Ei mice. Although it is necessary to generate TLR5-deficient mice by gene targeting to confirm the results, the report is interesting with regard to the role of TLRs other than TLR2 and TLR4.

In summary, TLR2 and TLR4 are essential for the recognition of distinct bacterial components. Whereas TLR2 recognizes PGN, LAM, zymosan and lipoprotein, TLR4 is required for LPS, Taxol and LTA signaling (Fig. 2). So far, structural similarity has not been found among the ligands of TLR2 or TLR4, indicating that additional molecules associated with TLR2 and/or TLR4 may determine the specificity of ligands. It is of note that when purified bacterial component preparations were used to investigate the TLR responsible, one should interpret the results carefully not to misread the effect of contaminating reagent. We believe that the usage of synthetic compounds, which mimic the structures of PAMPs, is the best way to prevent misunderstanding.

### 5. Signaling pathway through IL-1R/TLR

IL-1R family and TLRs, which feature a cytoplasmic TIR domain, are shown to share common signaling pathways. The presence of bacterial components such as LPS as well as IL-1 stimulation triggers the activation of transcription factors NF- $\kappa$ B and c-Jun N-terminal kinase, by which the transcription of proinflammatory cytokine genes occurs. The molecular events from the receptor to nucleus have been extensively investigated to identify signaling molecules required for the response.

Like IL-1R, TLR2 and TLR4 have been shown to activate IL-1R-associated kinase (IRAK) via an adaptor protein MyD88 [28,51–54] (Fig. 3). MyD88 possesses two remarkable domains, the N-terminal death domain and C-terminal TIR domain. Ligand binding to the IL-1R/TLR family results in the recruitment of MyD88 to TIR domains of receptors. The interaction of TLR and MyD88 is thought to be homophilic. The death domain of MyD88 is shown to interact with IRAK by homophilic interaction. IRAK is a serine/threonine kinase whose N-terminal portion harbors a death domain [55]. IRAK-deficient mice have been generated and showed reduced responses to LPS [56]. Subsequent to the activation of

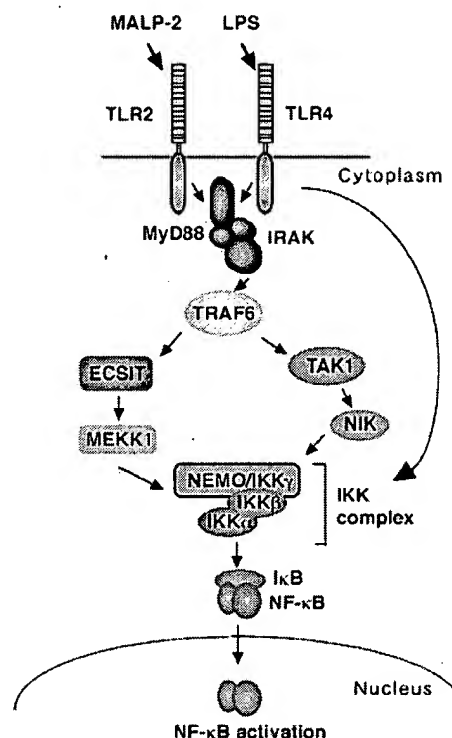


Fig. 3. Overview of TLR signaling pathways. The presence of bacterial components triggers the sequential activation of a signaling cascade leading to the nuclear translocation of NF- $\kappa$ B. MALP-2-induced NF- $\kappa$ B activation is fully MyD88 dependent, while LPS activates both MyD88-dependent and -independent pathways, indicating that there are some differences between the TLR2 and the TLR4 signaling pathway.

IRAK, another adaptor protein TNF receptor-associated factor (TRAF) 6 is phosphorylated and recruited to IRAK [57]. The C-terminal portion of TRAF6 shares homology with other TRAF family members and is known as the TRAF domain. This domain is shown to be required for oligomerization. In contrast, the N-terminal portion of this molecule is thought to be important for the activation of the downstream signaling pathway. Recently, Lomaga et al. [58] generated TRAF6-deficient mice. These mice were osteopetrotic, and the bone marrow macrophages derived from TRAF6-deficient mice showed impaired nitric oxide production in response to LPS. Further, defective activation of NF- $\kappa$ B in response to LPS, IL-1 and anti-CD40 was observed in TRAF6-deficient cells.

In resting cells, NF- $\kappa$ B associates with inhibitory I $\kappa$ B proteins, which sequester NF- $\kappa$ B in the cytoplasm. To release NF- $\kappa$ B from the inhibition of I $\kappa$ B, the phosphorylation of I $\kappa$ B by I $\kappa$ B kinases (IKKs) is required. The I $\kappa$ B phosphorylated by IKK complex undergoes polyubiquitination, and is degraded by proteasome. The kinase activity is found in a 700-kDa large protein complex and recently some components of it have been identified. These include two kinases (IKK  $\alpha$  and IKK  $\beta$ ) and a regulatory subunit (NEMO/IKK  $\gamma$ ) [59]. IKK  $\alpha$  and IKK  $\beta$  are highly homologous with each other, possessing N-terminal kinase domains followed by leucine-zipper and helix-loop-helix motifs. The critical role of IKK  $\beta$  and NEMO/IKK  $\gamma$  in TNF $\alpha$ , IL-1 and LPS-mediated NF- $\kappa$ B activation had been revealed by analyzing their knockout mice [59–62]. Embryonic fibroblasts from IKK  $\beta$ - and NEMO/IKK  $\gamma$ -deficient mice lacked NF- $\kappa$ B activation in response to TNF $\alpha$ , IL-1 and LPS. Both of these mutant mice died from E12.5 to E14 because of massive apoptosis in the liver. In contrast, IKK- $\alpha$  deficient mice died at birth with developmental defects in skin and limb [63–65]. IKK  $\alpha$ -deficient embryonic fibroblasts displayed normal NF- $\kappa$ B activation upon TNF $\alpha$  or IL-1 stimulation. IKK  $\alpha$  may be required for the regulation of unknown transcription factors, which govern limb and dermal differentiation.

Several molecules have been reported to relay the signal to IKK complex downstream of TRAF6 in the IL-1 and LPS signaling cascade. NF- $\kappa$ B-inducing kinase (NIK) is thought to directly interact with IKKs to activate them in response to upstream stimuli, such as LPS, IL-1 and TNF $\alpha$ . Overexpression of kinase-negative NIK inhibited TLR2- and TLR4-mediated NF- $\kappa$ B activation. TGF $\beta$ -activating kinase (TAK) 1 is also thought to be involved in the LPS signaling toward NF- $\kappa$ B [66,67]. It was reported that TAK1 associated with TRAF6 and acted upstream of NIK in IL-1-induced NF- $\kappa$ B activation. Overexpression of kinase-inactive TAK1 resulted in the impairment of NF- $\kappa$ B activation in response to IL-1 and LPS. The recently identified evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) is also implicated in the Toll/IL-1 signaling pathway [68]. ECSIT has been shown to interact with TRAF6 and activate NF- $\kappa$ B via MEKK1, which

is also a possible activator of NF- $\kappa$ B. However, further investigation is needed to confirm the *in vivo* role of these molecules in IL-1 and LPS signaling.

## 6. Critical role of MyD88 in IL-1R/TLR signaling

MyD88 is a cytoplasmic adaptor molecule essential for the signaling of the IL-1R/TLR family. As described above, MyD88 associates with a cytoplasmic tail of TLR/IL-1R upon stimulation with their ligand, and subsequently recruits IRAK to the receptor. We have previously generated MyD88-deficient mice and showed that MyD88 is essential for cellular responses to IL-1, IL-18. MyD88-deficient thymocytes lack an IL-1-mediated proliferative response and natural killer (NK) cells from MyD88-deficient mice did not show enhanced NK lytic activity in response to IL-18 [69]. In addition, MyD88-deficient mice were highly resistant to LPS-induced shock [70]. Macrophages derived from MyD88-deficient mice did not secrete TNF $\alpha$ , IL-6 and NO in response to LPS. B cells from the mice also showed a severely impaired proliferative response to LPS. Not only LPS, but also other bacterial components such as PGN, mycobacterial lysates and mycoplasmal lipopeptide MALP-2 failed to activate MyD88-deficient macrophages [49,71]. Therefore, we concluded that MyD88 is an essential mediator for the signaling of TLR2, TLR4 and IL-1R. Furthermore, we found that bacterial unmethylated DNA also elicits the activation of macrophages and B cells through a MyD88-TRAF6-dependent pathway [72]. In contrast, macrophages and splenocytes from TLR2- and TLR4-deficient mice responded normally to an oligonucleotide with a CpG motif. Hence, MyD88 may be also required for the signaling of TLRs other than TLR2 and TLR4. However, when we further investigated the signaling pathway in MyD88-deficient macrophages, we obtained surprising results. LPS-mediated activation of neither NF- $\kappa$ B nor JNK was abrogated in MyD88-deficient macrophages, except for a subtle delay of the activation [70]. Whereas wild-type macrophages showed both NF- $\kappa$ B and JNK activation within 10 min after LPS stimuli, MyD88-deficient cells took 20 min to confer NF- $\kappa$ B DNA binding activity and JNK activation. Surpris-

ingly, this delayed activation of transcription factors did not lead to the transcription of proinflammatory cytokine genes. These results imply that activation of additional mediators may be required for cytokine gene transcription. On the other hand, when MyD88-deficient macrophages were stimulated with a TLR2 ligand, MALP-2, they displayed neither NF- $\kappa$ B activation nor cytokine production [49]. These results indicate that there are differences in the signaling pathways in TLR2 and TLR4. In summary, TLR4, but not TLR2, has a MyD88-independent signaling pathway to activate the NF- $\kappa$ B and MAP kinase pathway in response to LPS.

## 7. Concluding remarks

Recent studies disclosed the mechanism of innate immune recognition via the TLR family. However, there remain many riddles to be solved. First, regardless of the importance of TLRs in bacterial recognition, it has not been reported that bacterial products directly bind TLRs. In addition, although *Drosophila* Toll has been reported to bind a ligand (Spätzle), a cognate ligand for mammalian TLR has not been identified. To clarify a precise mechanism of the PAMP recognition, additional molecules that form the PAMP receptor complex may need to be investigated. Concomitantly, investigation of the crystal structure of the TLR proteins will assist the understanding of precise mechanism of PAMPs recognition. Recently, Ohashi et al. [73] demonstrated that Heat-shock protein 60 (HSP60) is a putative ligand for TLR4. They showed that HSP60 elicited TNF $\alpha$  and NO production in peritoneal macrophages from C3H/HeN, but not C3H/HeJ mice. Although they did not show the interaction of HSP60 with TLR4, it is intriguing that an endogenous protein activates cells via TLR4.

It is not clear why more than 10 TLRs are encoded in the human genome. Further investigation of the function of TLRs other than TLR2 and TLR4 will clarify the entire mechanism of innate immune recognition. However, results from cells overexpressing TLRs sometimes mislead us as to the biological role of the molecule. We believe that the data based in overexpression experiments should be interpreted carefully. There is no doubt that a gene targeting

strategy is a potent way to analyze the function of these receptors.

Although the mammalian TLR signaling pathway mimics that of *Drosophila*, there are distinct differences between them. For instance, *Drosophila* induces specific and appropriate anti-microbial peptide in response to different microbial infections by activating different signaling pathways. In contrast, mammalian MyD88 plays a quite important role in the cytokine production in response to many bacterial components. Since an adaptive immune system exists in mammals, activation of nonspecific inflammatory responses and T cell instruction may be efficient for eliminating microorganisms. However, extensive investigation revealed that there are some differences in the intracellular signaling pathway between TLR2 and TLR4. NF- $\kappa$ B activation induced by MALP-2, a TLR2 ligand, is completely MyD88-dependent, whereas LPS has both MyD88-dependent and -independent signaling pathways leading to NF- $\kappa$ B via TLR4. Although the biological meaning of this MyD88-independent pathway is still to be clarified, it is possible that different PAMPs activate a modulated signaling pathway for the optimal responses to get rid of different microorganism even in mammals.

Lastly, Gram-positive and Gram-negative sepsis is one of the major causes of death even in the present day. Recent studies revealed that the TLR signaling pathway is a critical mediator of sepsis. An understanding of TLRs and their signaling pathway will reveal a therapeutic target for sepsis.

## Acknowledgements

This work was supported by grants from the Ministry of Education of Japan.

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## A CONSERVED SIGNALING PATHWAY: The *Drosophila* Toll-Dorsal Pathway

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KEY WORDS: Toll, Dorsal, NF- $\kappa$ B, I $\kappa$ B, dorsal-ventral patterning.

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### ABSTRACT

The Toll-Dorsal pathway in *Drosophila* and the interleukin-1 receptor (IL-1R)-NF- $\kappa$ B pathway in mammals are homologous signal transduction pathways that mediate several different biological responses. In *Drosophila*, genetic analysis of dorsal-ventral patterning of the embryo has defined the series of genes that mediate the Toll-Dorsal pathway. Binding of extracellular ligand activates the transmembrane receptor Toll, which requires the novel protein Tube to activate the cytoplasmic serine/threonine kinase Pelle. Pelle activity controls the degradation of the Cactus protein, which is present in a cytoplasmic complex with the Dorsal protein. Once Cactus is degraded in response to signal, Dorsal is free to move into the nucleus where it regulates transcription of specific target genes. The *Toll*, *tube*, *pelle*, *cactus*, and *dorsal* genes also appear to be involved in *Drosophila* immune response. Because the IL-1R-NF- $\kappa$ B pathway plays a role in vertebrate innate immunity and because plant homologues of the Toll-Dorsal pathway are important in plant disease resistance, it is likely that this pathway arose before the divergence of plants and animals as a defense against pathogens.

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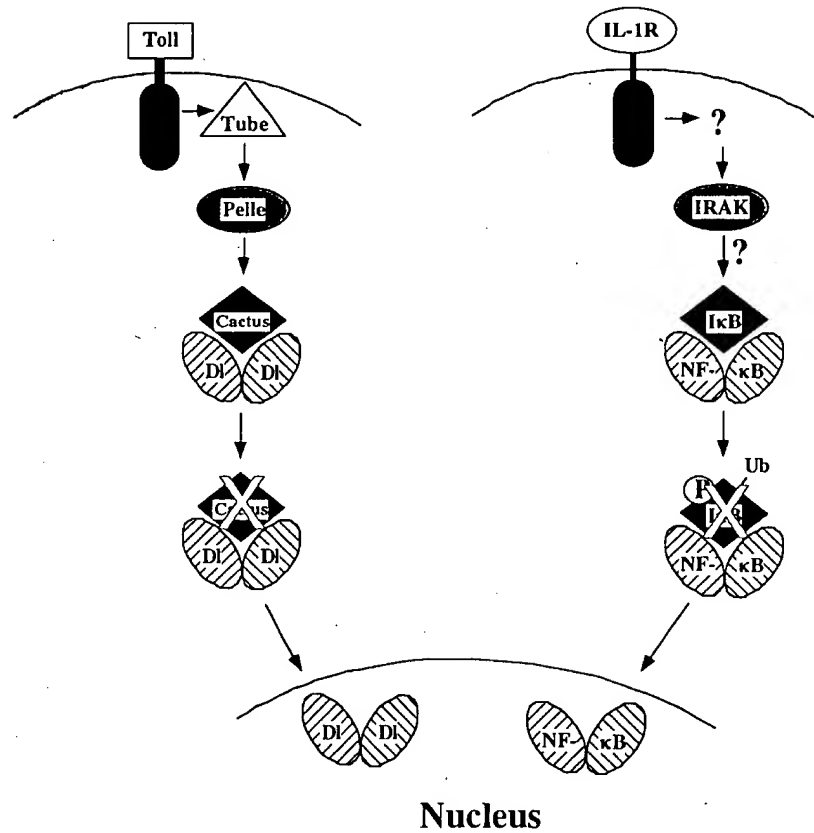
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## INTRODUCTION

Over the past few years, many examples of the evolutionary conservation of signal transduction pathways have been characterized. Through these studies it appears that although the biochemical interactions allowing signaling are frequently conserved, the same signaling pathway can mediate very different biological processes in different animals. The Toll-Dorsal pathway in *Drosophila* and the interleukin-1 receptor (IL-1R)-NF- $\kappa$ B pathway in mammals represent a conserved signaling pathway that is used in several, quite different biological contexts in both invertebrates and vertebrates. Dorsal and NF- $\kappa$ B are homologous transcription factors of the Rel family that are activated in response to binding of extracellular ligands to homologous transmembrane receptors, *Drosophila* Toll, and the mammalian IL-1R (Figure 1). A benefit of studying a signaling pathway that is conserved different species is the ability to exploit the strengths of each organism as an experimental system. In the case of the Toll-Dorsal and IL-1R-NF- $\kappa$ B pathways, the genetic experiments in *Drosophila* and biochemical experiments in mammalian cells contribute complementary sets of results that have accelerated the understanding of the pathway.

Because of its amenability to genetic analysis, the best characterized function of the *Drosophila* Toll-Dorsal pathway is the establishment of the dorsal-ventral pattern of the embryo (Anderson & Nüsslein-Volhard 1986, Morisato & Anderson 1995). Analysis of the effects of mutants on early embryonic patterning has defined the components of the signaling pathway and ordered the action of those components. Based primarily on expression data, the same pathway appears to be used in other processes at later developmental stages, including the *Drosophila* immune response (Ip & Levine 1994, Hoffmann 1995), morphogenetic movements (Gerttula et al 1988, Hashimoto et al 1991), and muscle development (Nose et al 1992, Halfon et al 1995).

In mammals, the function and regulation of NF- $\kappa$ B was first characterized in cultured cells. NF- $\kappa$ B is a dimer, most commonly a heterodimer composed



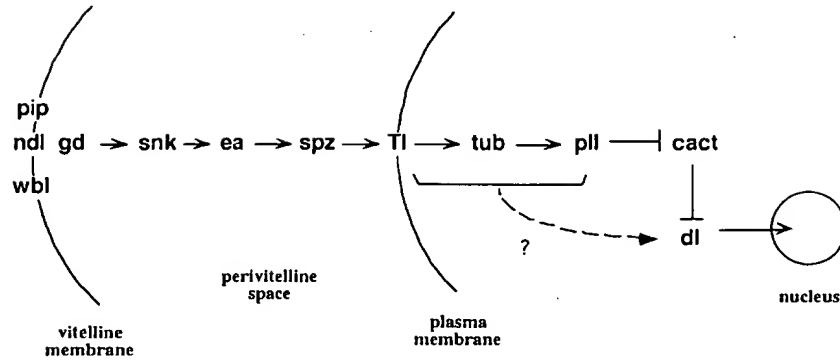
*Figure 1* Homology between the Toll-Dorsal and IL-1R-NF- $\kappa$ B pathways. The components of the cytoplasmic portion of the dorsal-ventral signal transduction pathway are homologous to components of the IL-1R-NF $\kappa$ B pathway. The cytoplasmic domains of Toll and IL-1R are homologous; Pelle, Cactus, and Dorsal are homologous to IRAK, I $\kappa$ B and NF- $\kappa$ B, respectively. NF- $\kappa$ B is a heterodimer of p50 and p65/RelA, whereas Dorsal exists as a homodimer. It is not known if IL-1R activates IRAK directly or through another protein, potentially a homologue of Tube. I $\kappa$ B is phosphorylated and ubiquitinated prior to its degradation; however, this has not been demonstrated for Cactus.

of p50 and p65 (RelA) subunits; other mammalian members of the family include p52, RelB, and c-Rel, which can associate in a variety of hetero- and homodimers (Siebenlist et al 1994). Biochemical studies have been particularly important in defining the biochemical mechanisms responsible for the activation of NF- $\kappa$ B (Baeuerle & Baltimore 1988, Ganchi et al 1992, Beg et al 1993, Traenckner et al 1994). Studies in cultured cell lines have implicated NF- $\kappa$ B in a variety of functions in the immune system, including the response to inflammatory cytokines, B cell and T cell proliferation, and B cell maturation (Siebenlist et al 1994). More recently, targeted mutations in the mouse genes have shown that particular NF- $\kappa$ B subunits are important in B cell maturation, T cell activation, and development of the fetal liver (Beg et al 1995b, Sha et al 1995, Weih et al 1995).

Thus far the only biological process common to *Drosophila* and mammals that uses the Toll-Dorsal/IL-1R-NF- $\kappa$ B pathway is innate immunity—the rapid nonspecific response to pathogens that leads to the production of antimicrobial peptides and cytokines, as well as activation of macrophage-like cells. This apparent conservation of function is made even more tantalizing by data showing that resistance to pathogens in plants involves proteins that are homologous to components of the *Drosophila* pathway (Staskawicz et al 1995). Innate immunity, therefore, may be the ancestral use of this signaling pathway, predating the divergence of plants and animals. In this context, it is interesting to speculate how the immunity function of this pathway was conserved, and how and when the pathway was co-opted for other biological functions.

### THE CYTOPLASMIC SIGNALING PATHWAY THAT INITIATES DORSAL-VENTRAL PATTERNING IN THE *DROSOPHILA* EMBRYO

The *Drosophila* genes that specify components of the Toll-Dorsal signaling pathway were identified because maternal effect mutations in these genes cause dorsalization of the embryo. Early in wild-type *Drosophila* development, cells adopt specific fates as a function of their dorsal-ventral position in the embryo: Ventral cells give rise to mesoderm, lateral cells give rise to the ventral nerve cord and ventral ectoderm, and dorsal cells give rise to dorsal ectoderm (Lohs-Schardin et al 1979). Twelve maternal effect genes, called the dorsal group genes, control the initial dorsal-ventral pattern of the embryo (Anderson & Nüsslein-Volhard 1984). Females that lack the activity of any one of eleven of these genes produce dorsalized embryos, in which cells at all embryonic positions give rise to dorsal ectoderm. The maternal effect phenotypes caused by these mutations are fully penetrant and constant from embryo to



**Figure 2** The dorsal-ventral signal transduction pathway. The twelve genes comprising the dorsal-ventral signal transduction pathway in *Drosophila* are listed in the order in which they are known to act. Germ-line clone analysis revealed that *pip*, *ndl*, and *wbl* are required in the soma. The other nine germ-line-dependent genes have been ordered with respect to each other and to the somatically required genes by genetic epistasis experiments. Abbreviations: *pip*, *pipe*; *ndl*, *nudel*; *wbl*, *windbeutel*; *gd*, *gastrulation defective*; *snk*, *snake*; *ea*, *easter*; *spz*, *spätzle*; *Tl*, *Toll*; *tub*, *tube*; *pll*, *pelle*; *cact*, *cactus*; *dl*, *dorsal*.

embryo, which has made genetic analysis of these components straightforward.

The order of action of the dorsal group genes has been defined by double-mutant analysis (Figure 2). Key in this analysis were the phenotypes of double mutants of dominant, constitutively active alleles of the *Toll* gene with loss-of-function mutations in other genes (Anderson et al 1985, Hecht & Anderson 1993, Morisato & Anderson 1994). These double mutants showed that activated *Toll* bypasses the requirement for seven genes; therefore these seven genes are normally required to activate *Toll* and lie upstream of *Toll* in the genetic pathway. These results also showed that three other genes, *tube*, *pelle*, and *dorsal*, were required for function of the activated *Toll*, placing these three genes downstream of *Toll*. The other key gene used in ordering the pathway was *cactus*. In contrast to the dorsalized phenotype caused by mutations in the other eleven dorsal group genes, loss-of-function mutations in *cactus* ventralize the embryo. Double mutants showed that *cactus* acts downstream of all the other maternal effect dorsal group genes except *dorsal*.

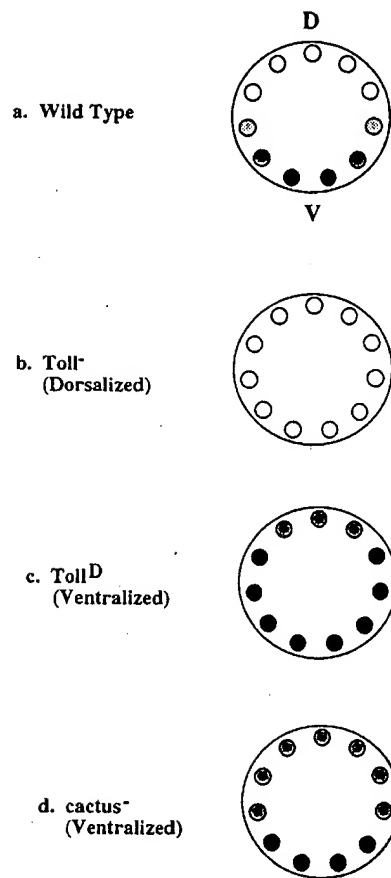
The molecular cloning and characterization of *Toll*, *tube*, *pelle*, *cactus*, and *dorsal* made it clear that these genes encode components of a signaling pathway that allows an extracellular signal to control the transcription of specific target genes in the embryo (Steward 1987, Hashimoto et al 1988, Letsou et al 1991, Geisler et al 1992, Kidd 1992, Shelton & Wasserman 1993). *Toll* encodes a

receptor protein with a single transmembrane domain; *tube* encodes a novel cytoplasmic protein; *pelle* encodes a cytoplasmic serine/threonine kinase; *cactus* encodes a cytoplasmic protein with an ankyrin repeat domain; and *dorsal* encodes a transcription factor related to the *rel* oncogene. From their order of action and their sequences, Toll appears to be a transmembrane receptor whose activation triggers a cytoplasmic signaling pathway involving Tube, Pelle, and Cactus, ultimately controlling the activity of the transcription factor Dorsal.

Analysis of the spatial distribution of the Dorsal protein revealed that the dorsal group genes control embryonic patterning by controlling the translocation of Dorsal protein from the cytoplasm to the nuclei on the ventral side of the embryo (Roth et al 1989, Rushlow et al 1989, Steward 1989). In very young wild-type embryos, Dorsal is distributed uniformly in the cytoplasm of the embryo. At the syncytial blastoderm stages, a nuclear gradient of Dorsal arises, with the protein most concentrated in the ventral nuclei of the embryo, less concentrated in lateral nuclei, and absent from nuclei on the dorsal side of the embryo (Figure 3a). In mutant embryos that are missing one of the dorsal group genes, the gradient of Dorsal does not arise. For instance, in embryos laid by *Toll*<sup>-</sup> females, Dorsal protein is present, but it is uniformly distributed in the cytoplasm and is not translocated into any embryonic nuclei (Figure 3b). The phenotype of *Toll*<sup>-</sup> embryos is exactly the same as the phenotype of embryos that lack Dorsal altogether; both kinds of embryos are completely dorsalized, indicating that Dorsal is active only when it is present in nuclei. Thus the dorsal-ventral pattern of the embryo is generated by a ventrally active signaling pathway that leads to a gradient of translocation of Dorsal protein into ventral and lateral embryonic nuclei.

Once in the nuclei, Dorsal acts as a transcription factor to define region-specific gene expression in the embryo. Dorsal activates the transcription of particular ventrally required genes, including *twist*, *snail*, and *rhomboid* (Pan et al 1991, Thisse et al 1991, Ip et al 1992a,b, Jiang et al 1992). The *twist* and *snail* genes are activated by the high concentrations of nuclear Dorsal protein found near the ventral midline of the embryo, which bind low-affinity Dorsal-binding sites in the promoters of the two target genes (Jiang & Levine 1993). The *rhomboid* gene is activated in a broad ventral and lateral domain where there are lower concentrations of nuclear Dorsal; transcriptional activation of *rhomboid* in lateral regions of the embryo requires cooperation between Dorsal protein and an unidentified, but apparently ubiquitous, basic HLH protein (Jiang & Levine 1993). Dorsal also represses the transcription of other genes in ventral cells in order to limit their expression to the dorsal side of the embryo; these genes include *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*) (Ip et al 1991, Schwyter et al 1995). Dorsal is converted from an activator to a repressor of transcription



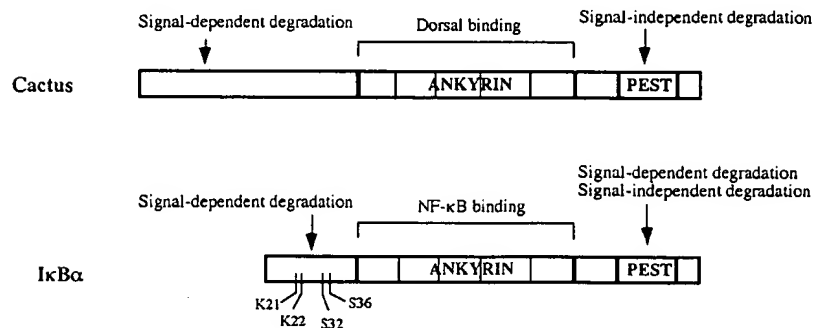


**Figure 3** The Dorsal gradient in wild-type and mutant embryos. The cartoons represent a cross section of a syncytial blastoderm *Drosophila* embryo, with the nuclei at the periphery of the embryo. The dorsal side is on the top (D) and the ventral side is on the bottom (V). (a) In wild-type embryos, nuclear Dorsal exists as a gradient, with high concentrations in ventral nuclei, and lower concentrations in lateral nuclei. On the dorsal side, all of the Dorsal protein is in the cytoplasm. (b) In embryos lacking Toll, no Dorsal protein enters the nuclei. (c) In embryos with a dominant allele of *Toll*, the Dorsal gradient is expanded. (d) In embryos lacking Cactus, Dorsal is nuclear all the way around the circumference, with the highest concentrations in ventral nuclei.

when it binds regulatory sites that are adjacent to binding sites for a corepressor, the HMG-like protein DSP1 (Lehming et al 1994).

### *Activation of Dorsal Protein Requires Disruption of the Cytoplasmic Cactus-Dorsal Complex*

In the oocyte and newly-laid embryo, the Cactus and Dorsal proteins are in a complex (Kidd 1992), with two molecules of Dorsal associated with one molecule of Cactus (Isoda & Nüsslein-Volhard 1994). Cactus is a *Drosophila* homologue of mammalian  $\text{I}\kappa\text{B}$  proteins (Haskill et al 1991), which form cytoplasmic complexes with NF- $\kappa\text{B}$  (Figure 4).  $\text{I}\kappa\text{B}$  family members and Cactus each contain a similar block of five to six ankyrin repeats in the middle of the protein. The ankyrin repeats of  $\text{I}\kappa\text{B}$  and Cactus mediate their interaction with Rel proteins (Kidd 1992, Hatada et al 1993, Jaffray et al 1995). The domains of Dorsal that interact with Cactus have been mapped (Isoda et al 1992, Kidd 1992, Norris & Manley 1992, Lehming et al 1995, Tatei & Levine 1995, Govind et al 1996). There is agreement that one region of the Dorsal Rel domain, including amino acids 233 and 234 (Lehming et al 1995), is crucial for Cactus binding. Based on the crystal structure of the Rel domain of p50, this site lies in a groove between the two p50 monomers, suggesting that Cactus binds in that groove (Kopp & Ghosh 1995, Müller & Harrison 1995). Some, but not all, assays indicate that two other regions of Dorsal are important for binding Cactus:



**Figure 4** A comparison of the Cactus and  $\text{I}\kappa\text{B}\alpha$  proteins. Cactus and  $\text{I}\kappa\text{B}\alpha$  each contain a set of five homologous ankyrin repeats in the center of the protein and a PEST sequence at the carboxy terminus. The ankyrin repeat domain of Cactus and  $\text{I}\kappa\text{B}\alpha$  is the site of interaction with Rel proteins. In Cactus, signal-dependent degradation is mediated by the amino terminus, whereas signal-independent degradation is mediated by the carboxy terminus. In  $\text{I}\kappa\text{B}\alpha$ , signal-dependent degradation is regulated by the amino terminus, and involves the phosphorylation of serines 32 and 36 and the ubiquitination of lysines 21 and 22. The carboxy terminus of  $\text{I}\kappa\text{B}\alpha$  has been implicated in both signal-dependent and signal-independent degradation.

the nuclear localization signal at the C-terminus of the Rel domain (Tatei & Levine 1995) and a region outside the Rel domain at the carboxy terminus of the protein (Isoda et al 1992, Norris & Manley 1992).

NF- $\kappa$ B proteins contain nuclear localization sequences that are masked by the binding of I $\kappa$ B (Ganchi et al 1992, Henkel et al 1992), providing an explanation for the cytoplasmic localization of these complexes. Similarly, it appears that Cactus masks the nuclear localization signal of the Dorsal protein, preventing its migration into nuclei. Consistent with this, in embryos that lack Cactus, Dorsal enters nuclei around the entire dorsal-ventral circumference of the embryo. Thus Cactus holds Dorsal in the cytoplasm until ventral signaling disrupts this complex, allowing Dorsal to move into nuclei on the ventral side of the embryo.

### *Cactus Protein Is Degraded in Response to Signaling*

Work on the mammalian homologue of Cactus raised the possibility that the signal-dependent disruption of the Cactus-Dorsal complex might involve degradation of Cactus protein. Several groups working in mammalian tissue culture cells showed that I $\kappa$ B is rapidly degraded after stimulation of responsive cell lines with interleukin-1 (IL-1), tumor necrosis factor alpha (TNF- $\alpha$ ), or phorbol myristate acetate (PMA), and that I $\kappa$ B degradation correlates with activation of NF- $\kappa$ B (Beg et al 1993, Henkel et al 1993, Miyamoto et al 1994).

In *Drosophila*, it is possible to activate the signaling pathway at a controlled time by injecting the ligand for Toll, activated Spätzle, into the extracellular space of the embryo (Schneider et al 1994). To test how signaling affects Cactus, high concentrations of activated Spätzle protein were injected into the extracellular space of embryos to activate the signaling pathway in all embryonic regions at a defined time (Belvin et al 1995). By analyzing Cactus protein on Western blots after injection, it was found that more than 90% of the Cactus protein in the embryo was rapidly degraded within 15 min after Spätzle injection. In embryos that lack Toll, Tube, or Pelle, the injection of activated Spätzle fails to cause Cactus degradation. Therefore, degradation of Cactus is a consequence of the activity of the Toll-Dorsal signaling pathway in the intact organism.

In theory, two models could explain the rapid degradation of Cactus in response to signaling: either signaling directly promotes Cactus degradation, or signaling causes separation of the Cactus-Dorsal complex and free Cactus is degraded. Taking advantage of *Drosophila* mutants, it was found that Cactus is rapidly degraded in response to signal even in *dorsal*<sup>-</sup> embryos, indicating that Cactus degradation is a direct consequence of signaling even when Cactus is not complexed with Dorsal (Belvin et al 1995). It is therefore likely that in the wild-type embryo, the activity of the signaling pathway targets the Cactus protein in the Cactus-Dorsal complex for degradation, liberating Dorsal from the complex and allowing it to move into embryonic nuclei (Figure 1).

*Toll, Tube, and Pelle Regulate Cactus Degradation*

Genes that act upstream of Cactus in the signaling pathway somehow target Cactus for degradation. The transmembrane protein Toll is activated on the ventral side of the embryo by binding a ventrally localized ligand, apparently a proteolytically processed form of the Spätzle protein (Morisato & Anderson 1994, Schneider et al 1994). The extracellular domain of Toll is composed largely of leucine-rich repeats, whereas the cytoplasmic domain of Toll is similar to the cytoplasmic domain of the mammalian IL-1 receptor (Schneider et al 1994). The sequence homology between the cytoplasmic domains of Toll and the IL-1 receptor appears to be significant because mutations that change amino acids conserved between the two proteins destroy receptor function (Schneider et al 1991, Heguy et al 1992). However, the biochemical mechanisms that convert ligand binding into activation of either receptor are not known.

Only two genes, *tube* and *pelle*, have been identified that act between Toll and the Dorsal/Cactus complex (Anderson et al 1985, Hecht & Anderson 1993). Analyses of double mutants using activated forms of *tube* and *pelle* have shown that *pelle* acts downstream of *tube* (Grosshans et al 1994, Galindo et al 1995). Activated *tube* and *pelle* were constructed in vitro by fusing the coding region of each gene with the extracellular and transmembrane domains of an activated allele of the *torso* receptor. Both the Torso-Pelle and the Torso-Tube fusion proteins promote ventral development, even in the absence of genes that act upstream of Toll. The *torso-pelle* construct promotes ventral development in the absence of *tube*, but the *torso-tube* construct has no activity in the absence of *pelle*, which places *pelle* downstream of *tube* in the signaling pathway.

*tube* encodes a novel 462-amino acid protein; the only striking sequence feature of the protein is the presence of five copies of an 8-amino acid repeat in its C-terminal half (Letsou et al 1991). Although these repeats are conserved in the *Drosophila virilis tube* gene, mutant forms of *tube* that lack the C-terminal 282 amino acids are still active in signaling, indicating that the N-terminal region of the protein is sufficient for function (Letsou et al 1993). Tube protein is highly enriched in regions near the plasma membrane of the syncytial embryo (Galindo et al 1995), where it might be involved in mediating signaling from Toll.

*pelle* encodes a cytoplasmic serine/threonine kinase (Shelton & Wasserman 1993). As Dorsal and Cactus are both phosphoproteins (Kidd 1992, Whalen & Steward 1993, Gillespie & Wasserman 1994), they are candidate substrates for Pelle. Bacterially produced Pelle is an active kinase but does not appear to phosphorylate either Dorsal or Cactus to a high degree (Grosshans et al 1994). However, Pelle can phosphorylate Tube in vitro, which raises the possibility of a dynamic interaction between Pelle and Tube proteins.

Genetic interactions between specific *pelle* and *tube* alleles suggest that the two proteins interact in vivo (Hecht & Anderson 1993). Direct interactions between the two proteins have been identified in the yeast two-hybrid assay (Grosshans et al 1994, Galindo et al 1995), where the N-terminal noncatalytic domain of Pelle interacts with the N-terminal essential domain of Tube (Galindo et al 1995). Because Tube is localized to the membrane, it is possible that activation of Toll causes Tube to recruit Pelle to the membrane, thereby activating Pelle. However, it is not yet known where Pelle protein is localized in embryos.

The homology of the Toll-Dorsal and the IL-1R-NF- $\kappa$ B pathways has motivated the search for mammalian protein kinases that act downstream of the IL-1 receptor and might be homologous to Pelle. A serine/threonine kinase, the IL-1 receptor-associated kinase (IRAK), that coimmunoprecipitates with the activated interleukin-1 receptor (M Martin et al 1994, Croston et al 1995) has been identified. Among all animal kinases, IRAK is most closely related to Pelle in sequence (Cao et al 1996). Within 30 s after treatment with IL-1, IRAK becomes associated with the IL-1 receptor and is extensively phosphorylated within minutes (Cao et al 1996). Thus because of both its biochemical behavior and its homology to Pelle, IRAK is an excellent candidate to mediate signaling from the IL-1 receptor to downstream cytoplasmic targets.

#### *Signal-Dependent Phosphorylation, Ubiquitination, and Degradation of I $\kappa$ B and Cactus*

In mammalian cells, I $\kappa$ B is transiently phosphorylated prior to its degradation (Beg et al 1993, Henkel et al 1993). Although this phosphorylation event is not sufficient to release NF- $\kappa$ B from the complex (Henkel et al 1993, Miyamoto et al 1994, Traenckner et al 1994, Alkalay et al 1995, DiDonato et al 1995, Lin et al 1995), phosphorylation of I $\kappa$ B appears to be necessary to target the protein for degradation (Traenckner et al 1994, Brown et al 1995, Chen et al 1995). Signal-dependent phosphorylation of Cactus has not been observed (Whalen & Steward 1993, Belvin et al 1995). However signal-dependent phosphorylation of I $\kappa$ B is difficult to detect because the protein is subsequently degraded within 1 to 2 min, so it is possible that Cactus is also phosphorylated in response to the signal but is degraded so rapidly that the phosphorylated form has not been detected. At this point, an attractive model is that Pelle phosphorylates Cactus, which targets Cactus for degradation. However, there are currently no biochemical data to support a direct interaction between Pelle and Cactus.

Site-directed mutagenesis that replaces either of two serines in the amino terminus of I $\kappa$ B (S32 and S36) with alanine prevents phosphorylation and degradation, suggesting that phosphorylation of these particular sites targets I $\kappa$ B for degradation (Brown et al 1995, Chen et al 1995), apparently by the

proteasome. The proteasome, a very large multiprotein structure found in all eukaryotes, is responsible for the degradation of cytoplasmic proteins that are marked by ubiquitination (Ciechanover 1994, Peters 1994). In the presence of peptides that inhibit the activity of the proteasome, ubiquitinated conjugates of I $\kappa$ B accumulate, with ubiquitin groups added to lysines 21 and 22 (Chen et al 1995, Scherer et al 1995). Because ubiquitinated forms of I $\kappa$ B do not accumulate in the S32A or S36A mutants, phosphorylation must precede ubiquitination. Therefore, the likely sequence of events in I $\kappa$ B degradation is signal-dependent phosphorylation of serines 32 and 36, followed by ubiquitination of lysines 21 and 22, followed by degradation by the proteasome.

Although the N-terminal domain of Cactus is much longer than that of I $\kappa$ B (Figure 4), there is some evidence that the N-terminal domain of Cactus is also required for signal-dependent degradation. A mutant allele of *cactus*, *cact*<sup>E10</sup>, lacks the N-terminal domain of the protein (N Ito, personal communication). The Cact<sup>E10</sup> product blocks nuclear localization of Dorsal, which suggests that it is not degraded in response to signal. Therefore, it is likely that the ability of Cactus to degrade in response to signaling depends on the N-terminal region of the protein.

Mutant alleles of a gene encoding one *Drosophila* proteasome subunit have been isolated (Saville & Belote 1993) and should provide a useful tool for analyzing whether the proteasome is important in the signal-dependent degradation of Cactus. Null alleles of this proteasome subunit gene are recessive lethal mutations, so partial loss-of-function alleles of the gene or genetic mosaics with mutant germ lines will be necessary to test whether the proteasome is required for Cactus degradation.

#### *Cactus Stability Is Regulated by Both Signal-Dependent and Signal-Independent Processes*

Both Cactus and I $\kappa$ B have PEST (Pro-Glu-Ser-Thr-rich) sequences in the region of the protein C-terminal to the ankyrin repeats (Figure 4). Because PEST sequences have been implicated in rapid protein turnover (Rogers et al 1986, Rechsteiner 1990), this region could be important for controlling the stability of these proteins. However, the PEST sequence in Cactus is not involved in signal-dependent degradation, because a mutant form of Cactus that lacks the entire C-terminus of the protein including the PEST region is degraded in response to signaling at approximately the same rate as wild-type Cactus protein (Belvin et al 1995).

Nevertheless, the PEST region is important for preventing the accumulation of free Cactus that is not complexed with Dorsal. Cactus is unstable in the absence of Dorsal protein (Kidd 1992, Whalen & Steward 1993) with the result

that all the Cactus protein in the wild-type embryo is complexed with Dorsal. In contrast, the mutant form of Cactus that lacks the PEST region is much more stable than wild-type Cactus in the absence of Dorsal (Belvin et al 1995) and, if present in sufficient amounts, can effectively block signaling and dorsalize the embryo (Belvin et al 1995). This result demonstrates the importance of maintaining controlled amounts of Cactus protein. It is not clear whether free Cactus blocks signaling because it competes with Cactus in the complex for the activity of the upstream signal or whether it acts by binding Dorsal rapidly after it is freed from the complex. Altogether, these studies show that two distinct regions of the Cactus protein mediate two different types of degradation: The N-terminus mediates signal-dependent degradation, and the C-terminus mediates signal-independent degradation. Both types of degradation are important for the proper regulation of signaling.

The C-terminal region of  $\text{I}\kappa\text{B}$  is probably also important for signal-independent degradation, because deletion of the C-terminal PEST-containing region increases the half-life of free  $\text{I}\kappa\text{B}\alpha$  (Miyamoto & Verma 1995). In contrast to Cactus, however, the C-terminus of  $\text{I}\kappa\text{B}$  appears to be required for signal-dependent degradation as well (Brown et al 1995, Rodriguez et al 1995). Because the C-terminus of  $\text{I}\kappa\text{B}$  appears to be involved in both signal-dependent and signal-independent degradation, whereas the domains of Cactus involved in the two kinds of degradation are physically separated, it may be easier to dissect these two kinds of regulation in *Drosophila* than in mammals.

#### *Parallel Signaling Events that Control Dorsal Activity*

The degradation of Cactus is the primary response to signal in the embryonic signaling pathway. However, embryos that lack all *cactus* activity still have a detectable dorsal-ventral asymmetry: These embryos have Dorsal protein in nuclei around the entire dorsal-ventral circumference, but there is more Dorsal protein in ventral than in dorsal nuclei (Roth et al 1991; A Bergmann, personal communication) (Figure 3*d*). Therefore, there must be some additional ventral signal promoting nuclear translocation of Dorsal protein that does not act through Cactus (Figure 2).

One possible parallel signaling event is the modification of Dorsal in direct response to signal. By comparing the Dorsal protein in mutant backgrounds that block or enhance signaling, it has been shown that nuclear Dorsal is more highly phosphorylated than cytoplasmic Dorsal (Whalen & Steward 1993, Gillespie & Wasserman 1994). However, it is not clear whether the increase in phosphorylation is a cause or an effect of the disruption of the Cactus-Dorsal complex. In embryos that lack Cactus and therefore have nuclear Dorsal protein around the entire embryonic circumference, a much larger fraction of the Dorsal protein is highly phosphorylated than in wild-type embryos, suggesting that free Dorsal

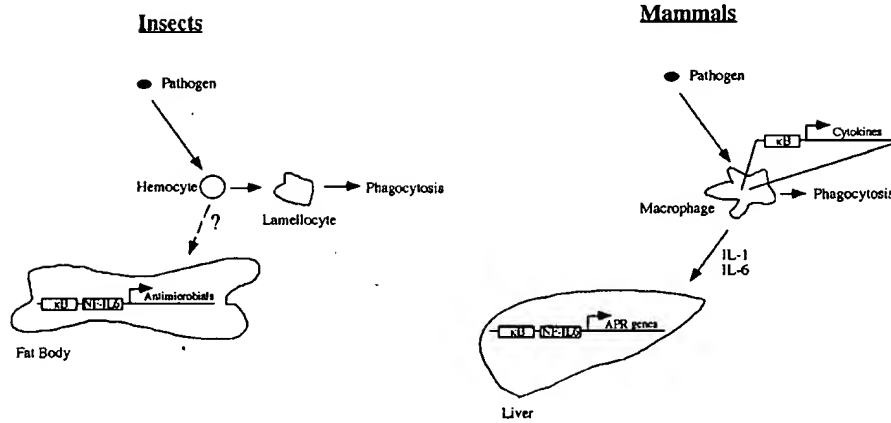


becomes highly phosphorylated independently of signaling. This interpretation is supported by the observation that *cactus*<sup>-</sup> embryos in which signaling is blocked by a mutation in an upstream gene also have a high level of highly phosphorylated Dorsal (Gillespie & Wasserman 1994). Thus once Dorsal is free from the Dorsal-Cactus complex, it becomes phosphorylated by a kinase whose activity does not depend on the signal. It is not known what sites in Dorsal become phosphorylated, nor is it known whether phosphorylation of those sites is important for Dorsal's activity as a transcription factor. However, it is interesting to note that in cultured *Drosophila* cells, mutation of a protein kinase A consensus phosphorylation site in the Rel domain inhibits both Dorsal nuclear localization and the ability of Dorsal to activate transcription of a reporter gene (Norris & Manley 1992).

Currently there is no evidence indicating that signaling directly increases phosphorylation of Dorsal. Therefore, some other mechanism may explain how, in the absence of Cactus, there is more Dorsal protein in ventral than in dorsal nuclei. One possibility is suggested by the recent report that at least one other *Drosophila* gene, *relish*, encodes a Cactus/I $\kappa$ B-like gene (Åslin et al 1995). The *relish* gene encodes a p105-like protein with both Rel and ankyrin repeat domains and is expressed maternally as well as later in development. It is possible (for the non-Cactus-mediated component of signaling) that Cactus is partially redundant and a small fraction of Dorsal is complexed with a different I $\kappa$ B family member, and that this I $\kappa$ B protein, like Cactus, is also degraded in response to signaling. Mutants in this gene may not have been recovered either because the maternal effect phenotype caused by loss of this minor component is very subtle, or because mutations in the gene cause lethality, which would prevent the recovery of maternal effect alleles.

## THE TOLL-DORSAL SIGNALING PATHWAY IN THE *DROSOPHILA* IMMUNE RESPONSE

In both insects and vertebrates, the rapid response to pathogens involves the activation of macrophages, which engulf invading pathogens, and production of antimicrobial peptides, which lyse invading pathogens (Figure 5). This process, called innate immunity, does not rely on specific recognition of pathogen and instead involves an immediate, general response to a wide range of pathogens without the subsequent development of specific long-term immunity (Sipe 1985, Hultmark 1993, Ip & Levine 1994, Hoffmann 1995). The first event in the innate immune response in mammals is the activation of macrophages, which engulf invading pathogens and secrete cytokines that activate other aspects of the immune response (Sipe 1985). Similarly, the hemocytes (blood cells) of insects



**Figure 5** Innate immunity in insects and mammals. In both insects and mammals, innate immunity involves the activation of macrophage-like cells and the subsequent production of antipathogenic agents in the liver, or its insect counterpart, the fat body. In *Drosophila*, hemocytes are activated in response to infection and differentiate into lamellocytes, a macrophage-like cell that can engulf invading organisms. The mechanisms leading to the induction of antimicrobial agents in the fat body are not known. In mammals, macrophages are activated by infection to produce inflammatory cytokines and to engage in phagocytosis. Through the secretion of IL-1 and IL-6, macrophages induce the transcription of acute phase response (APR) genes in the liver.

are activated by infection and participate in phagocytosis and encapsulation of invading pathogens (Götz & Boman 1985). The second major aspect of innate immunity is the production of the acute phase proteins by the liver and their secretion into the circulation; these acute phase proteins are involved directly in killing bacteria. Analogously, the fat body (the insect counterpart to the liver) produces and secretes a set of antimicrobial peptides in response to infection.

In mammals, NF- $\kappa$ B has been implicated in innate immunity in the response of macrophages to pathogens and the ensuing induction of acute phase proteins in the liver. The initial activation of macrophages by bacterial lipopolysaccharide triggers the activation of NF- $\kappa$ B and nuclear factor NF-IL6, which act synergistically to transcribe several of the cytokines (Matsusaka et al 1993). Among the cytokines secreted by the macrophages are IL-1 and IL-6 which, in addition to their role as inflammatory cytokines, induce the acute phase response in the liver (Koj 1985, Fey & Gauldie 1990). In addition, NF- $\kappa$ B and NF-IL6 synergistically activate many of these acute phase response genes (Betts et al 1993, LeClair et al 1992). The observation that the promoters of the *Drosophila* antibacterial genes also contain NF- $\kappa$ B and NF-IL6 sites sparked the interest in comparing insect immunity and the mammalian acute

phase response (Reichhart et al 1992). Data from several laboratories indicate that the Toll-Dorsal pathway is activated in the *Drosophila* immune response, but the precise function of these genes in immunity remains to be elucidated.

### *The Role of the Toll-Dorsal Pathway in the Humoral Immune Response*

The primary humoral response of *Drosophila* to infection is the induction of genes encoding antibacterial peptides in the fat body, the functional equivalent of the mammalian liver (Cociancich et al 1994). The first evidence suggesting that *dorsal* or a related gene might be important in the humoral immune response was the discovery of  $\kappa$ B binding motifs in the upstream regulatory regions of the genes encoding the antibacterial agents cecropin, diptericin, and attacin (Sun et al 1991, Reichhart et al 1992, Engstrom et al 1993, Kappler et al 1993). Biochemical experiments have shown that Dorsal binds  $\kappa$ B sites in the diptericin gene and activates a reporter construct carrying these sites (Engstrom et al 1993, Reichhart et al 1993). These observations led to the search for the expression of *dorsal* and *dorsal*-related genes expressed in the fat body that might activate transcription of the target genes in response to infection.

A gene closely related in sequence to *dorsal*, called *Dif* for Dorsal-related immunity factor, was cloned by homology to *dorsal* (Ip et al 1993). *Dif* is expressed at high levels during larval, pupal, and adult stages, but is not expressed during oogenesis or early embryogenesis (Ip et al 1993). Both *Dif* and *dorsal* are expressed in the larval fat body (Reichhart et al 1993, Lemaitre et al 1995), where they could mediate transcriptional activation of the antimicrobial peptide genes. *Dif*, like *Dorsal*, can bind the  $\kappa$ B sites in the cecropin gene and activate transcription in vitro, with *Dif* a more potent transactivator (Petersen et al 1995). At rest, both Dorsal and Dif are present in the cytoplasm of fat body cells. When larvae are infected by bacteria, Dorsal and Dif become localized to the nuclei within 30 min (Ip et al 1993, Reichhart et al 1993, Lemaitre et al 1995). However, the nuclear localization of Dorsal in response to signal is more complete: Essentially all the Dorsal moves into fat body nuclei, whereas the Dif response is more variable, and only about 30% of the fat body nuclei contain Dif after infection (Ip et al 1993, Reichhart et al 1993, Lemaitre et al 1995).

Other components of the Toll-Dorsal pathway play a role in the nuclear localization of both Dorsal and Dif in the fat body. By infecting larvae lacking components of the maternal dorsal-ventral pathway, it was determined that *Toll*, *tube*, and *pelle* are required for this nuclear translocation of Dorsal in the fat body (Lemaitre et al 1995). In addition, in larvae carrying *Toll* gain-of-function

or *cactus* loss-of-function mutations, Dorsal and Dif are constitutively nuclear in the fat body (Ip et al 1993, Lemaitre et al 1995). In contrast, components that act upstream of Toll in the dorsal-ventral patterning pathway, *spätzle*, *easter*, *snake*, and *gastrulation defective* have no effect on fat body nuclear localization of Dorsal. Therefore, it appears that only the cytoplasmic portion of the dorsal-ventral pathway is re-utilized in the humoral immune response, raising the question of what ligand(s) activate Toll in the fat body.

Despite the clear response of the Toll-Dorsal and Toll-Dif pathways to infection, their function in the fat body is unclear. In fat bodies of larvae lacking *Toll* or *dorsal*, cecropin and dipterecin are induced to wild-type levels (Lemaitre et al 1995), so Dorsal is not required for this aspect of the immune response. No mutation in *Dif* has been reported, thus it is not known whether Dif is important for induction of these peptides. It is possible that Dorsal and Dif are functionally redundant in the fat body and that only the double mutant will display a phenotype.

#### *Is There a Role for the Pathway in the Cellular Immune Response?*

There are also data suggesting that the Toll-Dorsal pathway is a component of the cellular immune response, although again, the exact function of the pathway is not clear. Larvae that lack *cactus* or that carry dominant constitutively active alleles of *Toll* have a high incidence of melanotic tumors. Melanotic tumors are not transplantable invasive growths, but instead are visible, melanized bodies that form as a normal part of the immune response and float in the larval or adult hemolymph. In the infection of a normal larva, the larval blood cells (hemocytes) change morphology from rounded cells to flattened cells (called lamellocytes) that encapsulate invading microorganisms and deposit melanin around the encapsulated pathogen. Melanotic tumor mutants have a high frequency of spontaneous melanotic tumors in the absence of infection, which apparently reflects abnormal activity of the hemocytes. Up to 50% of the hemocytes in larvae heterozygous for an activated *Toll* allele have the morphology of lamellocytes (Lemaitre et al 1995), suggesting that the spontaneous activation of blood cells may be responsible for the melanotic tumors. Because dominant *Toll* and loss-of-function *cactus* alleles share the same phenotype, it is likely that the signaling pathway controls hemocyte maturation and that constitutive activity of the pathway causes activation of unstimulated hemocytes. However, double-mutant larvae that lack both *dorsal* and *cactus* or only *dorsal* and carry a constitutively active allele of *Toll* still have melanotic tumors (Lemaitre et al 1995). Therefore, another target of the signaling pathway, besides Dorsal, must lead to activation of the blood cells.

## OTHER FUNCTIONS OF THE GENES OF THE TOLL-DORSAL PATHWAY IN THE *DROSOPHILA* LIFE CYCLE

Genetic and molecular data indicate that components of the Toll-Dorsal signaling pathway are used in a variety of other processes during the *Drosophila* life cycle, although it is not clear whether the entire pathway is used as a signaling cassette in any of these processes. The clearest indication that *Toll*, *tube*, *pelle*, and *cactus* are important in other processes is that null mutations in these genes decrease the probability of homozygotes surviving to adult stages. All animals that are homozygous for null alleles of *cactus* die during the pupal stage (Roth et al 1991); nearly all (> 95%) of the animals that are homozygous for null alleles of *Toll* die as larvae (Gerttula et al 1988); and only about 70% of animals homozygous for null alleles of *tube* or *pelle* survive to adult stages (Hecht & Anderson 1993). In contrast, animals homozygous for null alleles of *dorsal* are fully viable. Because mutations in the different genes have different effects on viability, it is clear that there must be differences in how these genes are used in later development. Either these genes have other functions unrelated to the signaling pathway defined in early embryos, or there are other genes expressed at later developmental stages that can partially substitute for components of these pathways. The functions of the Toll-Dorsal pathway in later developmental processes have been difficult to define. *Toll* is expressed in a dynamic pattern in many embryonic cells in the process of undergoing morphogenetic movements, which suggests that Toll might act as a coupled adhesion and signaling receptor. This is consistent with the observation that *Toll* expressed in *Drosophila* cultured cells promotes cell adhesion (Keith & Gay 1990). However, *Toll* mutants do not have gross defects in these morphogenetic movements. Mutants that lack *Toll*, *tube*, or *pelle* do form abnormally short pupae (Letsou et al 1991), indicating that these genes play a role in overall body shape, although it is not known what cell types control body shape.

*Toll* is expressed in a specific subset of developing muscles (Nose et al 1992, Halfon et al 1995). Embryos that lack *Toll* function have a variety of defects in muscle development, including muscle deletions, muscle duplications, and inappropriate muscle insertions (Nose et al 1992, Halfon et al 1995). The muscle phenotype is both variable and incompletely penetrant, suggesting that *Toll* overlaps in function with other genes affecting development of these muscles.

The only dorsal group gene that is completely lethal is *cactus*. Because flies that lack other components of the pathway are partially or completely viable, it may be that the lack of signaling via the Toll-Dorsal pathway is less harmful to the organism than is constitutive signaling. In a provocative parallel, mice that

lack *I $\kappa$ B $\alpha$*  die shortly after birth (Beg et al 1995a), whereas mice that lack p50 are viable. Interestingly, the lethality of *I $\kappa$ B $\alpha$*  mutant mice is suppressed by the concomitant loss of p50 (Beg et al 1995a), providing strong evidence that excess signaling in this pathway is more deleterious than lack of signaling. It will be interesting to test in *Drosophila* whether mutations in *dorsal* can suppress the lethality caused by *cactus* mutants, which would indicate that the lethality of *cactus* mutants results from excessive signaling through the pathway.

## CONCLUDING THOUGHTS

Recent work on the resistance of plants to pathogens has uncovered a remarkable similarity of plant disease-resistance genes (*R* genes) to components of the Toll-Dorsal signaling pathway (Staskawicz et al 1995). The *R* genes mediate the response of the plant to particular pathogens (gene-for-gene plant-pathogen interactions) and are thought to encode proteins that recognize pathogen and signal the immune response.

One set of *R* genes encodes proteins that are similar to Toll. Most dramatically, the tobacco *N* gene encodes a cytoplasmic protein with a domain homologous to the cytoplasmic domains of Toll and the IL-1 receptor, as well as a leucine-rich repeat domain similar to the Toll extracellular domain (Whitham et al 1994). *R* genes from other plants encode proteins with related but distinct domain structure. For instance, the tomato *Cf-9* gene encodes a transmembrane protein with extracellular leucine-rich repeats and a short cytoplasmic tail (Jones et al 1994), and the rice *XA21* gene encodes a transmembrane protein with extracellular leucine-rich repeats and a cytoplasmic serine-threonine kinase domain (Song et al 1995).

Another set of *R* genes, including the tomato *pto*, *pti*, and *fen* genes (GB Martin et al 1993, 1994, Zhou et al 1995), encodes cytoplasmic kinases that are more closely related to Pelle and IRAK than other kinases. The kinase domains of the tomato kinases and Pelle and IRAK are 30% identical to each other and define a subgroup of cytoplasmic kinases that we call the serine/threonine innate immunity kinases (the SIIK group).

The remarkable fact that related proteins are used in the signaling pathways that are activated by pathogens in mammals, insects, and plants makes it likely that these components were used in an innate immune response that evolved more than a billion years ago, before the divergence of plants and animals. As the components and steps in these signaling pathways become more completely understood, it may be possible to infer what the ancient forms of these signaling molecules looked like. For instance, was the original pathogen receptor a molecule like the rice *XA21*, with extracellular leucine-rich repeats to bind pathogen, and a cytoplasmic kinase domain to relay information to the inside

of the cell? If so, did a separate molecule like Pelle/IRAK later take over the kinase function of the pathway, allowing the receptor to diversify?

Even if we can deduce the nature of the ancient signaling pathway, the question of how this pathway was recruited to control embryonic patterning remains. Studies on early dorsal-ventral patterning in other insects and other invertebrates may shed light on how long ago this signaling pathway was adopted for use in embryonic patterning and could provide hints about the general problem of how signaling pathways are recruited for new functions.

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